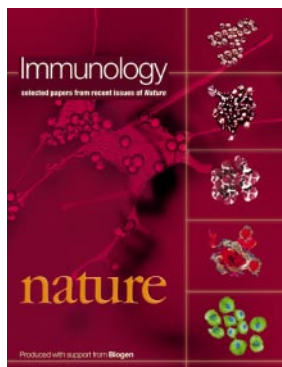


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Cover illustration

The transcription factor Pax5 determines B-cell lineage commitment by actively suppressing alternative lineage choices, so Pax5-deficient pro-B cells can take various differentiation pathways.

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We live in a hostile world. Our cells and tissues provide a rich environment for a multitude of infectious agents which are kept at bay only as long as we maintain an effective defence system. The study of the nature and regulation of this defence constitutes the field of immunology. Many pathologies involve the immune system either directly or indirectly, and often their treatments are also immune-based. Furthermore, the basic unit of immune function, the lymphocyte, is arguably the most well-studied of eukaryotic cells. Thus immunology is at the centre of biomedical science.

And yet it is a peculiarly inaccessible field. Historically, immunology has relied on concepts to rationalize the great complexity of responses. Although immunologists are eager to revise their approximations in the light of new data, this ever-changing landscape makes it difficult for non-experts to identify exactly where the field stands at any given time. We predict a growing appreciation of the subject as the molecular basis of immunological concepts become more clearly defined.

This special collection of immunology papers, all of which have appeared in our pages in 1999, presents the breadth of the field, and we are very pleased to acknowledge Biogen, whose financial support has helped to make this supplement possible. Indeed, *Nature* has published many of the landmark studies over the past 40 years and we aim to continue to be at the forefront. This collection emphasizes our commitment. But it also marks a growing commitment by the Nature Publishing Group as a whole, which has made the decision to launch a new monthly journal, *Nature Immunology*. Readers of *Nature* will be kept abreast of our plans in that direction. We hope that you find the papers in this supplement to be a stimulating appetizer for more top-quality immunology that *Nature* looks forward to publishing in the future.

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Dual personality of memory T cells

Charles R. Mackay

A newly identified chemokine receptor can identify two types of memory T cell with different migration preferences. Their existence may explain how memory cells initiate the rapid and effective secondary response to antigens.

Often, when a pathogen first infects the body, nothing happens — at least, not for several days. But when the same pathogen is encountered a second time, the response is rapid and vigorous. This phenomenon, known as immunological memory, is the basis for vaccination, yet very little is understood about how it protects the entire body so effectively. On page 34 of this supplement, Sallusto *et al.*¹ report that a newly identified cell-surface marker — the chemokine receptor CCR7 — can distinguish two subsets of T cells that carry immunological memory. These two subsets apparently circulate around the body by different pathways, and mediate the memory response in different ways. The results provide a new insight into the memory response, one that is relevant to basic immunology, as well as to vaccination strategies and the development of immunosuppressive therapies.

The immune system responds slowly to newly encountered pathogens owing to the relative lack of antigen-specific cells. New pathogens must first be transported to nearby lymphoid tissue, where the primary immune response develops and where naive lymphocytes and antigen-presenting cells interact. Two clonally expanded populations emanate from this primary response: 'effector' cells, which combat spread of the pathogen; and 'memory' cells, which guard against subsequent infections. Effector and memory cells are thought to be distributed to all tissues in the body, particularly epithelial surfaces (such as the skin and gut) where pathogens are likely to be re-encountered, so lymphocyte migration facilitates systemic immunological memory.

How can we tell the difference between naive, memory and effector T cells? This has been an elusive goal. In humans, isoforms of CD45 (a phosphatase involved in cell signalling) seemed able to distinguish naive and memory T cells. Any cell expressing the CD45RO isoform was regarded as a memory T cell, based on the fact that CD45RO⁺ (memory) T cells — but not CD45RA⁺ (naive) T cells — can respond vigorously *in vitro* to previously encountered antigens. Moreover, whereas CD45RA⁺ T cells migrate almost exclusively through lymphoid tissue², CD45RO⁺ T cells migrate throughout the body, including epithelial

surfaces. The rationale is that a naive T cell has such a low probability of seeing its specific antigen that it needs to travel through the lymph nodes, which are designed for massive migration of lymphocytes and antigen sampling². In contrast, memory or effector cells migrate mainly to peripheral tissues, providing protection at sites vulnerable to challenge by pathogens. But the problem with this system was that both memory and effector cells express CD45RO, so there was still no way to distinguish between the two.

Sallusto *et al.*¹ have now used a monoclonal antibody that recognizes the chemokine receptor CCR7 to discriminate between the two subsets of CD45RO⁺ T cells. The CCR7⁺ subset has many characteristics of effector cells. For example, these cells rapidly produce effector cytokines such as interferon- γ , interleukin-4 and interleukin-5, or they express

perforin granules (a feature of cytotoxic cells). Cells in the CCR7⁺ subset, on the other hand, behave more like true memory cells — they lack effector function, but the authors found that these cells could differentiate to CCR7⁺ effector cells after being stimulated with antigen. So, expression of CCR7, in conjunction with CD45RO, distinguishes memory cells from effector cells.

Many cells that migrate to the lymph nodes, such as naive T cells and antigen-presenting cells, express CCR7. Moreover, the chemokines that bind to CCR7 (MIP-3 β and secondary lymphoid chemokine; SLC) are both constitutively expressed in lymph nodes. For example, SLC is produced by specialized blood vessels in the lymph nodes and provides the entry cue for CCR7⁺ cells circulating in the blood³. And the lymphocytes in mice that lack CCR7 or SLC migrate poorly through the lymph nodes^{4,5}. But

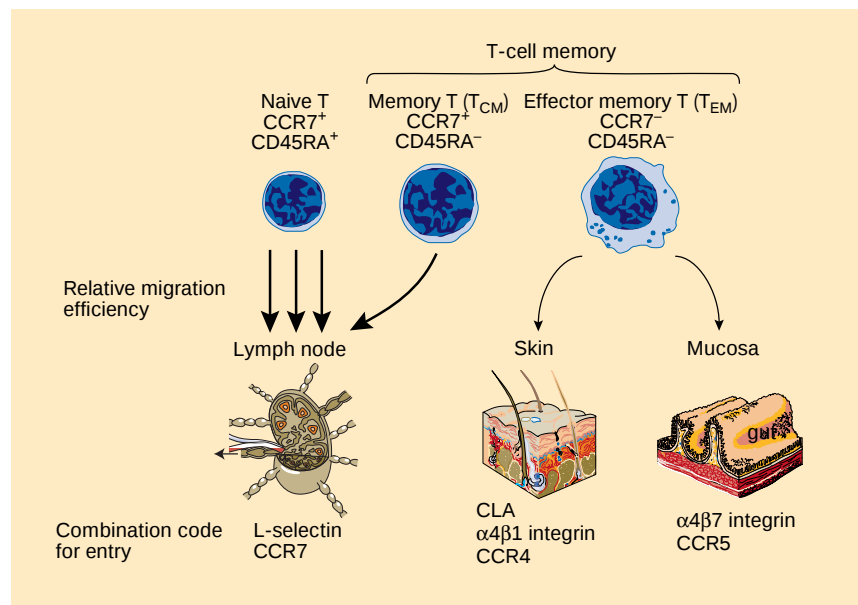


Figure 1 Two types of memory T cell with different migration preferences. Expression of CCR7 and the CD45RA isoform distinguishes three subsets of T cells: naive T cells (CCR7⁺ CD45RA⁺); a new subset, which Sallusto *et al.*¹ term central memory T cells (T_{CM}; CCR7⁺ CD45RA⁻); and effector memory T cells (T_{EM}; CCR7⁻ CD45RA⁻). Naive T cells migrate very efficiently through lymph nodes using a 'combination code' of L-selectin (an adhesion molecule) and CCR7 (a chemokine receptor). Central memory T cells probably migrate through lymph nodes, whereas effector memory T cells migrate through peripheral tissues such as the skin and mucosa. There are likely to be many combination codes for homing of T cells to the skin and mucosal tissues. CLA, cutaneous lymphocyte antigen.

CCR7 is only half the story, because cells first adhere to the walls of the lymph-node vessels using a molecule called L-selectin. So, the 'combination code' for entry of T cells to normal lymph nodes is L-selectin binding, followed by CCR7 signalling through SLC. This code is expressed by naive T cells and CCR7⁺ memory T cells (Fig. 1), but not by certain cells that do not recirculate, such as neutrophils or monocytes. Trafficking experiments show^{2,6} that a subset of memory T cells does indeed enter lymph nodes, although we cannot yet say whether these are Sallusto and colleagues' CCR7⁺ memory T cells.

The traffic of T cells through peripheral sites differs fundamentally from that through lymph nodes. So-called 'inflammatory' chemokines and receptors are involved, together with adhesion molecules that are upregulated by inflammatory cytokines. The T cells recruited to peripheral tissues are usually very distinctive, and include CD45RO⁺ T cells expressing chemokine receptors such as CCR5, CCR2 or CCR3 (ref. 7). But Sallusto *et al.*¹ show that these T cells, which are the effector-type cells, do not express CCR7. CCR5 is a particularly good marker for a subset of effector T cells, and T cells expressing CCR5 are prominent in inflammatory disease and at mucosal surfaces⁷ (a feature that may relate to the transmission of HIV-1). In fact, it is likely that the T cells that show a

degree of tissue-selective migration — preferential migration through the skin, say, rather than the gut — are effector-type T cells. For instance, the skin-homing subset of effector T cells expresses cutaneous lymphocyte-associated antigen and the chemokine receptor CCR4 (ref. 8).

The basis of immunological memory has long been contentious. According to one theory, the long-lived, recirculating memory T cells differentiate quickly to effector cells when they re-encounter an antigen. Another theory holds that constant exposure to the antigen is needed to maintain memory. Sallusto and colleagues now show that both CCR7⁺ (memory) and CCR7⁻ (effector) cells from people immunized with tetanus toxoid respond well to subsequent challenge with the toxoid *in vitro*. That is, toxoid-specific effector T cells remain alive and well, years after an initial immunization. The implication is that immunological memory is carried by both classical-type memory T cells (which Sallusto *et al.* term central memory T cells, T_{CM}) and effector T cells (now referred to as effector memory T cells, T_{EM})².

So what is the precursor-product relationship between the central memory T cell and the 'effector' memory cell? Sallusto *et al.* propose that the naive T cell differentiates to a central memory T cell and, finally, to an effector memory cell. A more conventional

idea is that effector cells are the precursors of memory cells⁹. Whatever the case, the existence of two types of memory cell with different migration pathways makes a lot of sense. Effector memory cells provide immediate front-line protection, particularly at epithelial surfaces. The central memory cells are the reserves, which can differentiate rapidly to effector memory cells in the lymph nodes in response to antigen. In conclusion, Sallusto and colleagues' study illustrates an important principle — the relationship between the functional activity of lymphocytes and their migration properties. For this reason, the chemokine receptors are proving to be excellent markers for various subsets of immune cells. ■

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Toll gates for pathogen selection

Richard J. Ulevitch

Two classes of innate immune receptors discriminate between different microbial pathogens to mediate host defence by mammalian macrophages.

All species need an immediate, systemic reply to the microbial pathogens in their environment. This reply — known as the innate immune response — is characterized by the *de novo* production of mediators that either kill the pathogen directly or induce phagocytic cells to ingest and kill it. In the fruit fly *Drosophila melanogaster*, distinct cell-surface receptors belonging to the Toll family mediate separate anti-bacterial and anti-fungal responses in the same type of cell¹. They do this by inducing genes that encode anti-microbial peptides.

Does this model describe the situation in mammalian systems? On page 39 of this supplement, Underhill *et al.*² describe the ligand specificity of two members of the mammalian Toll-like receptor family, TLR2 and TLR4. These data show that the situation is indeed similar to that in *Drosophila* — that is, two distinct Toll-like receptors, both expressed in macrophages, discriminate between different microbial pathogens or their products. Whereas TLR4 is the main protein involved in recognizing Gram-negative bacteria and lipopolysaccharide (a glycolipid constituent of the bacterial outer membrane), TLR2 is the key in responses to other types of microbial pathogen, such as yeast and Gram-positive bacteria.

The innate immune response is often driven through recognition, by the host cell, of surface components of the microbial pathogen. Monocytes and macrophages are central to the intensity and specificity of this response. Most — if not all — microbial pathogens are thought to activate the innate immune system using mechanisms with common features. For example, many macrophages contain a surface protein called CD14, which binds ligands such as lipopolysaccharide and triggers an innate immune response³. But CD14 is not thought to participate directly in signalling. Rather, one or more of the mammalian Toll-like receptors acts in concert with CD14 to discriminate between microbial pathogens or their products¹ and initiate transmembrane signalling.

Mammalian cells may express as many as ten distinct Toll-like receptors⁴. All span the cell membrane, with repeating leucine-rich repeats in the external domain and a common sequence motif — the Toll-homology

domain — in the cytoplasmic tail. Members of the interleukin-1 receptor family, which is another essential element of the innate immune system, also contain a Toll-homology domain in their cytoplasmic tails. Most attention has been paid to the TLR2 and TLR4 proteins, and the importance of TLR4 in responses to Gram-negative bacteria and lipopolysaccharide was first suggested by powerful genetic data. Beutler and colleagues⁵ established that TLR4 is encoded by the lipopolysaccharide (*Lps*) gene, and that it controls sensitivity to this molecule. Akira and colleagues⁶ also showed that mice in which the *TLR4* gene has been deleted have the same defects as mice with mutations in *Lps*.

But some reports indicate that lipopolysaccharide acts via TLR2. When TLR2 is expressed in cell lines that do not normally produce it, these cells become able to respond to lipopolysaccharide. Moreover, in at least one case, lipopolysaccharide-induced activation of a monocytic cell line is blocked by an anti-TLR2 monoclonal antibody⁷. So TLR2 has been considered a lipopolysaccharide receptor. But do these findings accurately reflect the physiological pathways of innate immunity?

These uncertainties can now be put aside thanks to the data from Underhill *et al.*² and to a report by Takeuchi *et al.*⁸ in this month's *Immunity*. These authors found that mice lacking TLR2 respond to lipopolysaccharide in the same way as do wild-type animals. They characterized responsiveness to lipopolysaccharide in both whole animals and macrophages. When the authors looked at macrophages from their TLR2-deficient mice, they found them to be less responsive than those from wild-type mice to cell-wall preparations from several distinct Gram-positive bacteria. These data not only support the idea that TLR4 is the essential element of a lipopolysaccharide-receptor complex that determines subsequent cellular responses to lipopolysaccharide, but they also point to a key role for TLR2 in innate immune responses to other microbial pathogens and their products. And although the initial findings implicating TLR2 as a lipopolysaccharide receptor seem unlikely to be physiologically relevant, they should nonetheless be recognized for having opened a new chapter

in innate immunity by showing that Toll-like receptors can activate cells in a ligand-specific way.

Where does this rapidly emerging field go now? Once again, studies in *Drosophila* may lead the way. For instance, Levashina *et al.*⁹ have highlighted the importance of proteases in controlling expression of the *spaetzle/Toll/cactus* genes in an anti-fungal defence system. This group showed that the absence of a serine protease inhibitor (serpin) encoded by the *Spn43Ac* gene results in constitutive activation of an innate immune response in the fly. Their findings support two main ideas: first, that the protease system activated by exposure to a microbial pathogen (or a product derived from that pathogen) is an essential control point in innate immunity; and second, that the Toll protein is not itself a pattern-recognition receptor in *Drosophila*¹⁰.

Given the parallels between the innate immune response in flies and in man, it is reasonable to look towards determining the ligand and specificity of all the Toll-like receptors. In mammals, are the pathogen-activated protease cascades represented by proteins in the complement and coagulation pathways? Or will careful analyses reveal that cell-surface protease systems work in concert with CD14 and members of the Toll-like-receptor family to generate innate immune responses? Whatever the case, such studies should uncover in more detail the conserved ancestral features of the two systems of innate immunity that are now being worked out in the *Drosophila* and mammalian systems. ■

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Selecting and maintaining a diverse T-cell repertoire

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To provide a T-cell population that will respond promptly to foreign antigen, the immune system looks inward, using the variety of self-antigens to select and maintain a diverse repertoire of receptors. A protective immune system must include a T-lymphocyte population that is poised to respond to foreign antigenic peptides presented by self-major histocompatibility complex molecules. As the organism cannot predict the precise pathogen-derived antigens that will be encountered, the system uses the diverse array of self-peptides bound to self-major histocompatibility complex molecules, not only to select a receptor repertoire in the thymus, but also to keep naïve T cells alive and 'ready for action' in the periphery.

The revelation that T lymphocytes recognize foreign antigen only when presented by major histocompatibility complex (MHC) molecules was made 25 years ago and remains the cornerstone of our understanding of cellular immunity¹. The nature of antigen presentation was unknown for more than a decade until it became clear that foreign antigen has to be processed into peptides that can bind stably to the groove of an MHC molecule to allow recognition by the T-cell receptor (TCR)^{2,3}. MHC genes are extremely polymorphic and the various allelic products bind a broad spectrum of different peptides, explaining MHC restriction of T-cell recognition. During T-cell development, immature T cells are chosen to mature on the basis of the ability of their clonally expressed TCR to interact weakly with self-MHC molecules expressed in the thymus. During this process of positive selection, self-peptides bound in the MHC groove are also recognized. Thus, the selection of the TCR repertoire is not based solely on the recognition of one or a few self-MHC structures, but on the recognition of those structures modified by the binding of an enormous diversity of self-peptides. Low-affinity interactions between the newly expressed TCRs and self-peptide-MHC molecules select a diverse repertoire that will be fine-tuned to react strongly to pathogen-derived peptides bound to the same MHC in the periphery. Even after maturing and leaving the thymus, T cells continue to depend on survival signals transmitted by the interaction of the TCR with self-peptide-MHC complexes. The long-term homeostasis of naïve T cells depends on continuous tickling of the TCR, and the rebound of the size and complexity of the peripheral lymphocyte pool after any marked loss in cell numbers involves recognition of self-peptide-MHC complexes. Infection often results in massive clonal expansion and development of effector function that initially overrides the balance of lymphocyte pools. When the pathogen is cleared, balance is restored: effector cells are eliminated and a long veteran's retirement is guaranteed for the surviving memory cells, which are no longer dependent on recognition of self-antigen. The veterans may be recalled to action quickly by a subsequent infection with the same pathogen. To provide protection against both unknown new pathogens and the recurrence of old infections, the immune system must maintain separate populations of naïve cells and memory cells. In addition, the system must retain an appropriate mixture of CD4⁺ helper T cells and CD8⁺ cytotoxic T cells in both naïve and memory pools.

Thymocyte development

Bone marrow stem cells enter the thymus and commit to the T lineage in response to signals from the microenvironment⁴. The stages of development of the major lineages of T cells are delineated by the surface expression of the co-receptor molecules CD4 and

CD8. The earliest precursors are CD4⁺CD8⁺ double negative (DN) in phenotype, comprising about 3% of the total thymocyte numbers. These proliferate extensively and become CD4⁺CD8⁺ double positive (DP) cells, which are still immature and are the targets for TCR-based selection events. The MHC molecules expressed by the epithelial elements of the thymus are critically involved in the process of positive selection, which allows a small fraction of the DP population to mature into single positive CD4 lineage or CD8 lineage T cells^{5,6}.

Assembling the TCR

As is the case for antibodies, functional TCR genes are assembled from separate V, D and J region segments by genetic recombination⁷. For T cells expressing $\alpha\beta$ receptors, this process of gene rearrangement occurs in the thymus. Mice and humans carry in their germline a large number of V gene segments, which encode roughly the first 90 residues of a mature TCR α or TCR β chain. The TCR β chain gene is assembled at the DN stage of T-cell development. A short D gene segment is juxtaposed to a short J segment and this is followed by rearrangement of a V segment to the assembled downstream DJ segment. In the case of TCR α chains which rearrange later at the DP stage there are no D segments; a large number of V α segments may rearrange to a large number of J segments. The immunoglobulin-like fold of the TCR chains positions three loops or complementarity determining regions (CDRs) from each chain in close proximity to each other, creating a binding face that will contact antigen. Two of these CDR loops, namely 1 and 2, are encoded by the V gene segment and have only the diversity provided by the number of germline V region gene segments, around 20–70 V α and V β segments in mice and humans. The third CDR loop is created by the juxtaposition of VJ or V(D)J segments and provides much more diversity, a result of the ability of each V segment to rearrange to any (D)J segment compounded by the fact that the joining of the coding sequences is imprecise. Nucleotides may be added or deleted at each of these junctions. If there were only 50 V α and 50 V β genes to encode the TCR repertoire, combinatorial pairing would provide only $50 \times 50 = 2,500$ TCRs. The addition of (D)J segments and the imprecision of joining boosts this number into the billions, with sequence diversity concentrated in the CDR3 loops.

TCR interaction with ligand

Crystal structures of MHC class I and class II molecules have revealed their mode of function (Fig. 1). Although differing in detail, both MHC class I and class II molecules have peptide-binding grooves to present antigen to T cells^{8–10}. In each case, the groove is made up of a 'floor' of β -sheets and 'walls' of long

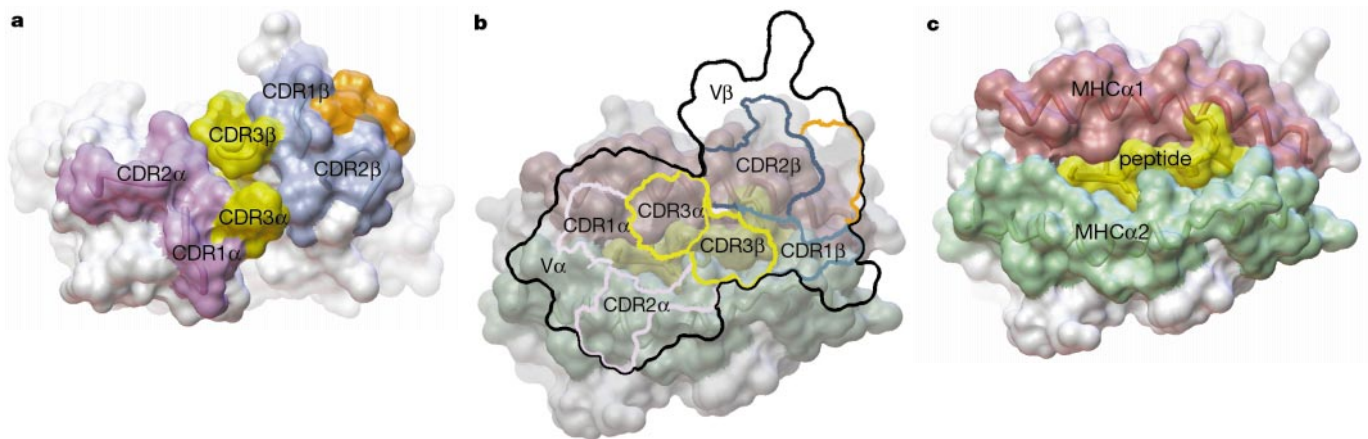


Figure 1 The nature of TCR interaction with peptide-MHC ligand. **a**, Positioning of the three CDR loops of the TCR α and TCR β chains in the ligand-binding face of the TCR. **b**, Footprint of the TCR on the peptide-MHC complex. **c**, MHC class I molecule with bound

peptide. The long α -helices of MHC enclose the peptide. Reprinted with permission from Garcia *et al.*¹¹. The crystal structure of a TCR interaction with a MHC class II ligand has not been reported but is likely to follow the same rules.

α -helical regions. The most polymorphic residues in the MHC molecules protrude into the groove, thus altering the spectrum of peptides that can be bound. The remarkable feature of MHC-peptide binding is that it is both extremely stable yet promiscuous, allowing many different peptides to bind each allele. Many of the stabilizing interactions are between the backbone of the peptide and the MHC, whereas a few pockets in the groove select for certain side chains on the so-called 'anchor residues' of the peptide. MHC class I molecules load peptides soon after synthesis in the endoplasmic reticulum before trafficking to the cell surface. In contrast, the groove of MHC class II molecules is protected from peptide loading until the molecules reach endocytic vesicles. Once loaded with a peptide derived from lysosomal processing, the class-II-peptide complex, like the class-I-peptide complex, is expressed stably at the cell surface.

Mature T cells discriminate between self and foreign by recognition of peptides presented in the groove of self-MHC molecules. The membrane-distal face of the peptide-MHC complex that is presented to the T cell consists of a flat surface made up principally of the α -helices of the MHC surrounding a central peptide. High-resolution structures of the TCR complexed with peptide-MHC ligand have shown how this is achieved (Fig. 1). Crystal structures of a murine TCR interacting with MHC class I and peptide^{11,12}, and two human TCRs complexed with the HLA-A2 class I molecule bound to the same viral peptide^{13,14} have been solved, and all of the structures showed the same basic topology of interaction¹¹⁻¹⁶. The TCR is positioned in a diagonal orientation over the face of the peptide-MHC complex with the CDR1 and CDR2 loops of the TCR α chain lying over the N-terminal half of the peptide and the corresponding regions of the TCR β chain lying over the C-terminal half. Many of the interactions of the V gene segment-encoded CDR1 and CDR2 regions of the TCR are actually with MHC residues. In particular, the CDR1 and CDR2 loops of the V α chain have almost identical sites of interactions with peptide-MHC in all structures solved so far, and this match-up may be critical in establishing the overall configuration. The most variable loops of the TCR, namely the CDR3 regions of the TCR α and TCR β chains, lie centrally and have most contact with protruding peptide side chains. Thus, the most variable region of the TCR (CDR3 loops) has best access to the most variable region of the ligand (peptide).

The unselected TCR repertoire

The lymphoid-specific components of immunoreceptor gene rearrangement, the recombina-activating gene (RAG) products, are first expressed in thymocytes soon after entry in the thymus and

operate initially on the TCR β loci. Successful VDJ rearrangement resulting in the production of a functional, in-frame TCR β chain is tested by assembly of the new gene product (TCR β) along with an invariant surrogate TCR α chain, pre-T α , plus signalling components of the TCR, the CD3 complex¹⁷. Proper assembly of this complex with the newly generated TCR β chain signals the CD4⁺CD8⁺ pre-T-cell to switch off expression of RAG proteins, to go through several rounds of division and to proceed to the CD4⁺CD8⁺ stage of thymocyte maturation. This check point, called β -selection, does not involve the recognition of any ligand by the pre-TCR, as truncated versions of TCR β and pre-T α lacking the extracellular domain can trigger progression to the DP compartment¹⁸. The mere assembly of the CD3-pre-T α -TCR β complex appears to be sufficient to activate the downstream signalling pathway. It is possible, although perhaps unlikely, that some TCR β chains are lost in this selection if they cannot assemble with CD3 and/or pre-T α .

At the DP stage of thymocyte differentiation, the RAG genes are expressed once again, and the cells now focus on rearranging TCR V α to J α . Production of a new TCR α chain that can pair with the existing TCR β chain allows surface expression of the TCR $\alpha\beta$ -CD3 complex. Co-expression of randomly rearranged TCR $\alpha\beta$ at the DP cell surface is an expression of the germline, or unselected TCR repertoire. The repertoire that is allowed to leave the thymus for the peripheral lymphoid organs is only a subset of this larger pool. To complete maturation, the germline repertoire is positively and negatively selected for interactions with MHC molecules in the thymus. Ultimately, only about 5% of the DPs are allowed to emigrate. In a murine thymus that lacks expression of any MHC molecule, T-cell development is blocked at the DP stage. DP thymocytes from such a MHC-deficient environment are immature and unresponsive to conventional antigen, yet tricks have been used to study the specificity of their germline TCRs. In one case, maturation of the thymocytes was induced in foetal thymic organ culture by receptor engagement with anti-TCR antibodies¹⁹. The mature CD4⁺ T cells that developed were cloned as hybridomas to allow analysis of their TCR specificity. In another case, DP thymocytes from MHC-deficient mice were isolated and reaggregated with MHC wild-type thymus stromal cells. In such reaggregate cultures, the fraction of cells that respond to MHC class I or MHC class II could be scored by examining the upregulation of surface markers that are normally turned on during thymocyte selection²⁰. Both of these approaches showed that the germline TCR repertoire is skewed to the recognition of MHC molecules. Somewhere between 5 and 20% of the DP population from MHC-deficient mice was responsive to the set of MHC molecules expressed in any one mouse

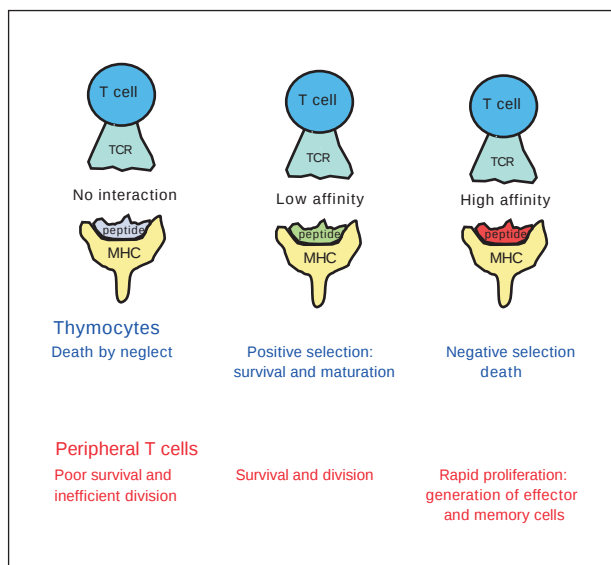


Figure 2 Consequences of TCR interactions with ligands of varying affinity in the thymus and periphery. TCR–MHC interactions may facilitate maturation, survival, proliferation or death. The consequences of TCR binding to peptide–MHC are dictated by the affinity of the interaction, as well as the maturation state of the T cell. Immature thymocytes require a positively selecting signal, which is delivered through low-affinity interactions of their newly rearranged TCR with self-peptide–MHC. If no signal is delivered to the thymocyte because of an inability of the TCR to interact with self-peptide–MHC complexes, it will undergo programmed cell death known as death by neglect. Alternatively, a thymocyte may express a TCR that has high affinity for self-peptide–MHC. These cells must be eliminated by negative selection as they are potentially autoreactive. In the periphery mature T cells require low-affinity interactions with self-peptide–MHC to ensure survival. Furthermore, low-affinity interactions with self-peptide–MHC allow naïve T cells to expand when T-cell numbers are reduced. High-affinity interactions between a naïve T-cell in the periphery and peptide–MHC typically initiate the immune response against a foreign antigen. This leads to rapid proliferation and the development of the effector function culminating in the emergence of a few antigen-experienced cells that join the pool of memory T cells to await a subsequent encounter with antigen.

strain. This is a much higher frequency than would be expected by the generation of receptors through the random rearrangement of gene sequences that do not have an inherent predisposition for interactions with MHC. Evolution has shaped the TCR genes, presumably the CDR1 and CDR2 regions encoded by the V gene segments, so that their $\alpha\beta$ product will preferentially match MHC molecules.

Certain V α gene segments have evolved to interact preferentially with either class I or class II molecules. TCRs bearing these V α segments are preferentially selected into the CD8⁺ (MHC class I reactive) or CD4⁺ (MHC class II reactive) T-cell subset during normal thymocyte selection. Swapping individual residues in the CDR1 or CDR2 regions of the V α gene is sufficient to change the selection of these TCRs from the CD4⁺ to the CD8⁺ subset²¹. Furthermore, certain mouse strains differ consistently in the ratio of CD4⁺ to CD8⁺ T cells in peripheral organs, and one of the loci controlling this phenotype maps with the TCR α locus²².

Positive selection of thymocytes

Even though they express a TCR at the surface, in the absence of MHC recognition CD4⁺CD8⁺ thymocytes will undergo apoptotic death because of receptor neglect (Fig. 2). During their approximate 3-day life span, the DP thymocytes will continue to express the RAG genes and continue to rearrange TCR α chain genes. TCR engagement with MHC class I or class II molecules on the cortical thymic epithelial cells is required to rescue the cell from death by neglect and to switch off further TCR α chain gene rearrangement. Only

those DP thymocytes that bind self-MHC with sufficiently low affinity will be rescued. In contrast, DP cells that express a receptor that interacts with high affinity with MHC in the thymus will be eliminated in a process referred to as negative selection. Almost half of the cells reacting with self-MHC are lost through this mechanism²³ (Fig. 2). The low-affinity recognition of self-MHC not only promotes thymocyte survival but also determines commitment of the DP thymocyte to either the CD4 or the CD8 lineage^{5,6,24}. Recognition of MHC class I results in commitment to the CD8 lineage, whereas recognition of class II results in CD4⁺ T cells. Thus, function and lineage are correlated with MHC class recognition. How positive selection on MHC class I versus class II is distinguished by the thymocyte is unknown. Lineage choice may be steered by qualitative or quantitative differences in signalling, or may be random but confirmed by later selective events.

The molecular nature of the TCR–MHC recognition event in positive selection has been an area of active research. Initial experiments showed that mutations affecting only the inside of the groove of a murine class I molecule drastically altered the selected repertoire of CD8⁺ T cells^{25,26}. These experiments suggested strongly that self-peptides bound to the MHC groove are recognized by thymocytes and are critical for positive selection. Further analysis of this recognition required the use of an *in vitro* organ culture system, using thymus lobes from mice deficient in class I expression in which peptides could be added back to reconstitute positive selection. Disruption of the β_2 -microglobulin gene, encoding the light chain of class I molecules, or of the TAP genes encoding a transporter that delivers peptides to nascent class I molecules in the endoplasmic reticulum, leads to a drastic decrease in selection of CD8 lineage cells. Surface expression of MHC class I can be restored to some extent by the addition of single peptides or peptide mixtures to organ culture systems. Initial experiments using thymic lobes from mice that express a normal, germline TCR repertoire suggested that a complex mixture of peptides restored selection of CD8⁺ T cells better than single peptides which bound and stabilized MHC to the same degree^{27,28}. Follow-up experiments in this peptide add-back system using mice that express rearranged TCR transgenes of known ligand specificity suggested a stringent requirement for peptide recognition in the positive selection of CD8 lineage T cells^{29–31}. Peptides that bind MHC class I and form a low-affinity ligand for the TCR induced the maturation of functional CD8⁺ cytotoxic T cells most efficiently³². The measured TCR affinities for foreign peptide–MHC ligands fall into the 1–10 μ M range³³. Compared with this, the affinities for ligands that can efficiently induce positive selection, but cannot induce negative selection, are only 5–9-fold lower³⁴.

Is a single self-peptide actually responsible for the maturation of a cohort of thymocytes? Or do a larger number of self-peptides select each TCR? Natural peptides eluted from MHC class I molecules can induce selection of certain TCR transgenic thymocytes when added back to TAP-deficient thymus lobes^{35,36}, although whether these are expressed at the appropriate level in the thymus cortex is not known. Antibodies that react in a peptide-specific fashion with only a subset of class II molecules prevent the thymic selection of a specific subset of CD4⁺ T cells³⁷. Certainly, it can be concluded that a subset of self-peptides, and possibly a single self-peptide, bound to self-MHC acts as the ligand that permits the maturation of each T cell. In this way, these self-peptides act as low-affinity mimics or 'stand ins' to prime the immune system to interact later in the periphery with high-affinity ligands for the TCR such as foreign peptide bound to the same MHC³⁸.

In the study of CD4⁺ T-cell selection in the thymus, researchers have generated mice in which all or a large fraction of the class II molecules in an individual mouse are occupied by the same peptide, and have asked how many T cells can one peptide–MHC ligand select. The invariant chain Ii is a chaperone for MHC class II that contains a region, CLIP, that occupies the class II groove in the

endoplasmic reticulum and prohibits other peptides from binding until the molecules reach endosomal/lysosomal vesicles. At this site, CLIP is removed by the action of the peptide exchange catalyst, H2-M. Mice deficient in H2-M express near normal levels of class-II-peptide on their surface, but in this case, most remain occupied by the CLIP peptide^{39–41}. Another strategy has been to generate a covalently linked class-II-peptide construct that assembles with the single peptide 'sewn in' to the MHC groove and to express these as transgenes in mice that lack wild-type MHC class II^{42,43}.

Initial results from these 'single peptide' systems were remarkable in showing that the mice developed a large and broad CD4⁺ T-cell repertoire. They contained 20–50% of the normal numbers of CD4⁺ T cells, with diverse TCRs as assessed by V β and V α usage. Could a single peptide-MHC complex select a near-normal CD4⁺ T-cell repertoire? More recent analysis has shown quite clearly that the repertoire in these animals is far from normal. First, none of the single-peptide mice can positively select TCRs of known specificity that are selected in normal mice^{44–46}. Second, closer analysis of the TCR α chain usage in the mutant versus wild-type mice shows that the selected repertoire is markedly constrained in the mutants⁴⁷. Last, even when 95% of class II molecules are occupied by a single peptide, most of the CD4⁺ T cells that emerge from the thymus are actually selected on the 5% of molecules that are occupied by a diverse mixture of unknown self-peptides. The CLIP region of Ii was replaced with a tight-binding peptide, so that in mice that were Ii-deficient, 95% of surface MHC class II molecules were occupied by the substituted sequence⁴⁸. Under these conditions, large numbers of CD4⁺ T cells were selected. The critical experiment was to breed these mice to an H2-M-deficient background which resulted in no apparent change in the level of MHC class II expression, but a 70% decline in the number of CD4⁺ T cells selected. The simple conclusion is that the 'background' of diverse peptides bound to class II must have a major role in selection even though such complexes are present at extremely low levels.

Positive selection of immature DP thymocytes by low-affinity interactions with self-peptide-MHC complexes implies that cells at this stage of development are more sensitive to low-affinity ligands than are their mature descendants. This has been shown to be the case, despite the fact that they express 10-fold less TCR on their surface^{49,50}. Low-affinity ligands stimulated early activation events in DP thymocytes but not in mature T cells bearing the same TCR; however, from what we have learned recently about the homeostasis of peripheral T cells, we might assume that under certain circumstances, mature T cells can also respond to low-affinity self-ligands.

Peripheral T-cell homeostasis

After the selection and maturation of T cells in the thymus, naïve T cells enter the periphery where they circulate throughout the secondary lymphoid organs awaiting an encounter with antigen. The organization of the many cell types that make up the immune system requires complex homeostatic mechanisms to assure the production and survival of each cell type in appropriate numbers to maintain proper immune function. One can imagine many different mechanisms by which appropriate homeostasis of lymphocyte populations is maintained, including control of the rate of production, division of mature cells, trafficking and cell death.

To maintain homeostasis of lymphocyte populations, the different cell types must be grouped and counted. Peripheral B- and T-cell populations appear to be independently regulated⁵¹. This conclusion is based on the fact that similar numbers of mature B cells are found in the periphery of normal mice and in mice that lack T cells⁵². Likewise, the number of T cells found in normal mice does not increase in B-cell-deficient mice⁵³. In addition, when small numbers of T cells are introduced into depleted hosts, they undergo expansion equivalently in mice deficient in both B and T cells and in mice deficient only in T cells⁵⁴. All these results suggest the independent homeostatic control of T-cell and B-cell numbers.

The pool of peripheral T cells consists of naïve T cells and previously activated antigen-experienced memory cells. Interestingly, naïve and memory T cells appear to occupy separate homeostatic niches. After reconstitution of T-cell-deficient mice with memory cells, the transferred cells expand to equal the same total number of memory cells in normal mice. Furthermore, it is not possible to increase the size of the memory pool by transferring large numbers of memory T cells, indicating that there is a set point for memory T-cell number even when no naïve T cells are present⁵⁵. The separate regulation of memory and naïve pools makes teleological sense as it is vital to retain both a diverse naïve repertoire that can respond to invasion by yet unknown pathogens and a memory pool that can respond rapidly to previously encountered pathogens. If these two populations were in competition for survival, the diverse repertoire of naïve T cells could be replaced by rapidly expanding memory T cells or, conversely, new thymic emigrants could displace memory T cells resulting in the loss of immunological recall⁵¹.

Although CD4⁺ and CD8⁺ cells serve different functions in the immune system and recognize different MHC molecules, many results suggest that they occupy overlapping homeostatic niches⁵¹. First, relatively normal numbers of total T cells are found in mice deficient in MHC class I or II or in the CD8 or CD4 co-receptors, and thus lacking one T-cell subset. Second, small numbers of mature peripheral T cells will expand when introduced into a T-cell-deficient host, and the co-transfer of large numbers of either CD4⁺ or CD8⁺ mature T cells can suppress this expansion^{56,57}. Last, in TCR transgenic mice in which the T-cell population is skewed toward either the CD4⁺ or CD8⁺ subset, total T-cell numbers are typically near normal. Thus, CD4⁺ and CD8⁺ T cells appear to be counted together for purposes of maintaining the homeostasis of T-cell numbers. However, some distinction is clearly made between CD4⁺ and CD8⁺ T cells, as the ratio of CD4⁺ to CD8⁺ T cells is tightly regulated in normal mice and will rapidly readjust after transfer of large numbers of either subset⁵⁶.

The size of the naïve pool of T cells remains more or less constant in spite of the continuing contribution of recent thymic emigrants, the loss of T cells due to normal cell death, the expansion and waning of effector populations during immune responses, and the diversion of cells into the memory pool after an immune response. The size of the peripheral T-cell pool does not appear to influence the rate of thymocyte production or export of naïve T cells, as changes in the number of peripheral T cells do not change the rate of thymocyte output⁵⁸. These data suggest that homeostatic control over the size of the naïve peripheral pool is regulated by the loss of T cells in the periphery⁵⁸. How recent thymic emigrants are incorporated into the peripheral pool is not entirely understood. Clearly, recent thymic emigrants must displace other naïve T cells to maintain homeostasis of the peripheral T-cell pool. Newly generated T cells could either randomly displace resident T cells in the periphery or selectively displace resident cells on the basis of a criterion such as age^{51,58,59}. Even in the absence of thymic export, the naïve pool remains rather constant in size, indicating that the life span of naïve T cells is under homeostatic control^{59,60}.

Relatively little is known about the mechanisms that determine life span, capacity for renewal and survival of naïve T cells in the periphery in the absence of antigen. Naïve T cells were considered to be quiescent 'spore-like' cells that persist indefinitely without antigenic stimulation^{61–65}; however, evidence now suggests that naïve T cells may require input signals to support their survival in the periphery. Expression of transcription factors such as lung Kruppel-like factor and NFAT4 are crucial for the maintenance of a resting, naïve population of T cells^{66,67}. In addition, cytokine signals influence the survival, homing and activation of naïve T cells, which in turn alter the activation state and homeostasis of the naïve compartment^{68,69}. The bcl-2 family of molecules, which are intimately involved in the survival of many cell types, are also involved in the maintenance of the naïve pool of T cells⁷⁰. Now it

appears that TCR interactions with self-MHC direct a signal required for maintenance of naïve T cells.

MHC in peripheral maintenance

Although self-peptide-MHC molecules are known to be crucial in the generation of a T-cell repertoire with a wide spectrum of specificities, only recently have data emerged that implicate TCR interaction with self-MHC in the survival and expansion of peripheral T cells (Fig. 2). Several groups have shown that MHC molecules are important in maintaining a peripheral pool of T cells⁷¹.

In many experimental systems, naïve CD4⁺ T cells demonstrate a requirement for MHC class II molecules for long-term survival^{72–75}. CD4⁺ T cells were positively selected in MHC class-II-expressing thymuses and then assayed for survival in a periphery that was engineered to lack expression of the positively selecting MHC class II allele. In all cases, CD4⁺ T cells declined in numbers in the absence of self-MHC class II. One report has shown that expression of MHC class II on dendritic cells is sufficient to support CD4⁺ T cells in the periphery⁷⁴.

An important role for MHC class I in maintaining naïve CD8⁺ T cells has been demonstrated by the transfer of TCR transgenic CD8⁺ T cells into MHC class-I-deficient hosts^{76,77}. Both studies showed that the naïve CD8⁺ T cells fade away in hosts that lack peripheral expression of MHC class I. Furthermore, the MHC class I allele that positively selected the T cell in the thymus is strictly required for naïve T-cell survival⁷⁶. The inability of CD8⁺ T cells to accumulate in mice expressing very low levels of MHC class I when grafted with a wild-type thymus further supports a role for TCR engagement by MHC in the periphery to permit survival of T cells⁷⁸. Although some differences were reported in these studies, the trend remains clear: the retention of both CD8⁺ and CD4⁺ T cells is enhanced when MHC class I and II, respectively, are expressed in the periphery.

MHC in recovery of peripheral homeostasis

The naïve T-cell pool consists mostly of resting cells compared with the memory pool in which cells turn over more rapidly⁶³. Upon close examination, however, low levels of division are observed in naïve T-cell populations in the absence of conventional antigen stimulation^{61,63}. Furthermore, even without conventional antigen stimulation, naïve T cells have a great potential for division when transferred into hosts with low numbers of lymphocytes^{56,60,62,65,79–83}. This proliferation of naïve cells after transfer into lymphopenic hosts has been speculated to involve cytokine release, the expression of environmental antigens or interactions with self-MHC molecules^{57,65,82,84–86}. Recently, the molecular interactions that support the expansion of naïve T cells in lymphopenic environments have become of significant interest, and several groups have studied the function of MHC in this process. There is a clear consensus that both CD4⁺ and CD8⁺ T cells require expression of MHC ligands for efficient homeostatic expansion in lymphopenic hosts^{54,57,77,86–89}.

When polyclonal or TCR transgenic monoclonal CD4⁺ T-cell populations are transferred into syngeneic hosts that have been T-cell depleted, they divide without direct antigenic stimulation. If the CD4⁺ cells are transferred into class II-deficient hosts or hosts expressing MHC class II other than the positively selecting class II molecule, however, little division is observed^{54,57,86,87}. Similarly, both polyclonal and TCR transgenic CD8⁺ T cells expand in wild-type hosts expressing MHC class I, but minimally in hosts with low or no levels of MHC class I molecules^{77,88,89}. Thus, both CD8⁺ and CD4⁺ T cells proliferate much more efficiently in a lymphopenic host expressing MHC class I or MHC class II molecules, respectively. These data show that MHC recognition contributes to division of naïve T cells responding to homeostatic signals.

What is the specificity of the TCR-MHC interaction that promotes homeostasis-driven expansion? Experiments comparing CD4⁺ T cells transferred into hosts with wild-type self-peptide-

MHC class II or hosts expressing MHC class II bound to a limited repertoire of self-peptides show that CD4⁺ T cells undergo expansion efficiently only when transferred into a host with a self-peptide-MHC class II repertoire from the background in which the T cells developed. Division of wild-type CD4⁺ T cells was observed in T-cell-depleted wild-type hosts but only minimally in H2-M-deficient hosts. As expected, CD4⁺ T cells that were positively selected in the H2-M-deficient background were able to divide when transferred into T-cell-depleted H-2M-deficient hosts^{57,86}. These data make a strong case that peptide-specific recognition of MHC class II complexes by T cells promotes division in T-cell-depleted environments^{57,86}. Furthermore, they suggest that this recognition is specific for the same peptides that T cells previously recognized with low affinity during their positive selection in the thymus^{57,86}.

CD8⁺ T cells also exhibit a requirement for specific recognition of peptide-MHC class I complexes to undergo homeostatic expansion. Proliferation of TCR transgenic CD8⁺ T cells in TAP-deficient hosts expressing low levels of MHC class I was restored by the addition of a single MHC class-I-binding peptide⁸⁹. Recognition was peptide specific, as a low-affinity peptide known to positively select the TCR transgenic T cells promoted expansion, whereas a control peptide did not. Thus, the signal for homeostatic proliferation may be characterized by low-affinity TCR interactions with self-peptide-MHC complexes. This suggests that the specific recognition of self-peptide-MHC complexes drives homeostatic proliferation of peripheral T cells, and that this interaction has a specificity similar to the recognition of self-peptide-MHC during positive selection. It is possible that the positively selecting peptide(s) may not necessarily be expressed in the periphery, but that other ligands of similar affinity or avidity for the TCR function in this capacity (Fig. 2).

The response to T-cell depletion

To what extent does homeostasis-driven proliferation of peripheral T cells in a self-peptide-MHC-specific manner contribute to a useful immune system? One possibility is that it ensures the diversity of the peripheral T-cell repertoire. It is appealing to consider that a requirement for peptide-specific recognition during homeostatic proliferation would reinforce the requirement for self-recognition established during positive selection for an individual clone to survive over time and contribute to the long-lived pool of peripheral T cells. Furthermore, the ability of a naïve T cell to receive TCR-self-peptide-MHC signals may determine to what extent a given cell contributes to reconstitution after a decline in T-cell numbers. This could have important implications for peripheral homeostasis after a loss of peripheral T cells in adults, as the ability of the thymus to replenish a naïve T-cell pool with diverse specificities may be insufficient to repopulate the periphery efficiently⁸⁴. The clinical relevancy of such a mechanism certainly exists for patients with T-cell immunodeficiencies resulting from infection (such as AIDS) or from therapeutic treatments (such as chemotherapy or irradiation).

Until recently, it was thought that naïve T cells did not recognize self-peptide-MHC ligands in the periphery. However, the existence of mechanisms for MHC-dependent homeostasis-driven proliferation and survival suggest that low-affinity interactions between the TCR of peripheral T cells and self-peptide-MHC complexes are involved in regulating homeostasis of peripheral T-cell populations. T cells proliferate in response to low-affinity self-ligands when T-cell numbers are reduced, which demonstrates that the sensitivity of the TCR or the response to TCR-mediated signals can be altered by global homeostatic signals. We can envisage at least three different mechanisms by which this could work (Fig. 3). Under normal conditions, T cells crowding through lymph nodes may be competing for ligands on presenting cells. Competition may be for MHC itself or for some other growth factor; lymphopenia would decrease

the competition. Alternatively, T cells may 'count' their own numbers by sending inhibitory signals to each other, or by inhibiting the stimulatory properties of a presenting cell. Decreasing T-cell numbers would sensitize the T cell or activate the presenting cell (Fig. 3).

One implication of naïve T cells being induced to proliferate in T-cell-depleted environments is that what has previously been considered 'maintenance' is likely to be the sum of survival and proliferation. The decline of T cells in the absence of MHC may be partially due to their inability to undergo homeostatic proliferation. Thus, it is necessary at this point to re-evaluate our understanding of T-cell maintenance to accommodate a more complex regulation of T-cell homeostasis.

Maintenance of the memory T-cell pool

Memory T cells often divide in the periphery long after antigen stimulation, even without evidence for the persistence of antigen. Furthermore, the rules for the persistence of memory T cells appear to be different from those that naïve T-cells obey. Recent studies have shown that CD4⁺ and CD8⁺ memory T cells have a much less stringent requirement for TCR–MHC interactions for survival and homeostatic proliferation compared with naïve T cells.

The survival of memory CD8⁺ T cells is much less dependent on MHC class I than that of naïve CD8⁺ T cells. Both polyclonal and TCR transgenic memory CD8⁺ T cells specific for lymphocytic choriomeningitis virus (LCMV) survive indefinitely in MHC-

deficient hosts⁷⁷. Memory CD8⁺ T cells, expressing the TCR transgene specific for the male antigen HY, survived and proliferated without a preference for the restricting allele⁷⁶. However, unlike the LCMV-specific memory T cells, the memory T cells specific for the HY antigen disappeared after transfer into mice expressing low levels of MHC class I^{76,90}. In both experimental systems, CD8⁺ memory T cells were able to proliferate in lymphopenic hosts even when MHC class I was not expressed, showing that homeostatic proliferation of memory CD8⁺ T cells is MHC class I independent^{76,77}. Although some differences in conclusions from these studies may be attributed to distinct experimental systems, the data with polyclonal CD8⁺ T cells specific for many LCMV antigens present a convincing argument for MHC independence for homeostatic proliferation and survival of memory CD8⁺ T cells⁷⁷. Similarly, memory CD4⁺ T cells survive indefinitely in hosts that do not express their positively selecting MHC class II molecule⁹¹ or any class II molecules at all⁹². Thus, CD4⁺ memory T cells appear to have no requirement for the presence of self-MHC class II to support their long-term survival. An obvious corollary of these observations is that neither CD8⁺ nor CD4⁺ memory T cells need continuous exposure to their cognate antigen to survive, a subject of much previous debate.

The response to infection

There is concern that certain infectious agents may overwhelm the immune system because of their high replicative potential; however, the opposite problem may arise. Analyses have shown that, in some cases, the specific immune response can threaten to overwhelm the lymphoid compartment. Use of peptide–MHC tetramers to stain T cells on the basis of TCR specificity, and of single-cell assays to detect interferon- γ secretion, have revolutionized our appreciation of the numerology of the CD8⁺ T-cell response to infection^{93,94}. For mice infected with the natural pathogen LCMV, total CD8⁺ T-cell numbers increase 2–10-fold by eight days after exposure (Fig. 4). Remarkably, most of the CD8⁺ T-cell expansion is antigen-specific! The rare, antigen-specific, naïve CD8⁺ T cells present before infection can divide 13–16 times in this time, so that their frequency may go from 1 in 10⁴–10⁵ to making up most of the CD8⁺ T-cell population. Such rapid proliferation leads to the production of

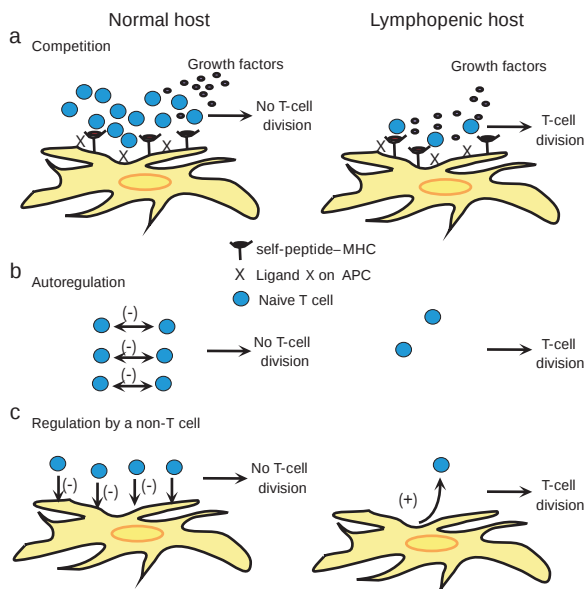


Figure 3 Possible mechanisms to explain the proliferation by naïve T cells in response to low-affinity ligands following T-cell depletion. The lymphopenic state renders naïve T cells capable of responding by proliferation to peptide–MHC complexes that are not stimulatory during normal conditions. **a**, Competition for niches or space on the antigen-presenting cell (APC) would be reduced when T-cell numbers are depleted, thereby allowing greater accessibility to ligands or growth factors. Such decreased competition for non-MHC-derived stimulatory signals such as growth factors or ligands could make signals accessible that would allow T cells to detect self-peptide–MHC complexes resulting in proliferation. It seems improbable that a decrease in direct competition for peptide–MHC complexes explains the ability of T cells to proliferate in response to self-peptide–MHC ligands because CD4⁺ and CD8⁺ T cells recognize distinct ligands yet appear to occupy largely overlapping homeostatic niches. **b**, T cells could somehow 'sense' a drop in T-cell number. T cell/T cell interactions could inhibit proliferation or decrease sensitivity to TCR signals. A release from autoinhibition during lymphopenia would allow T cells to receive a signal for proliferation following interactions with low-affinity ligands for the TCR. **c**, T-cell loss could be detected by another cell type that would upregulate the expression of a factor promoting T-cell division by influencing the T-cell response or sensitivity to TCR interactions with low-affinity peptide–MHC ligands.

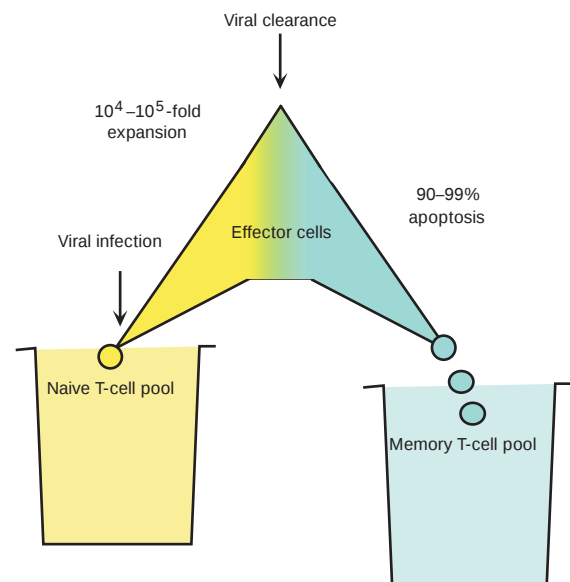


Figure 4 Response to infection and the recovery of homeostasis. The case of the murine CD8⁺ T-cell response to LCMV is depicted. After viral infection, naïve CD8⁺ T cells that recognize LCMV epitopes may go through 13 or more divisions within 8 days, producing massive numbers of effector cells. When antigen is cleared, most of the effector cells die, leaving an expanded population of LCMV-specific T cells, which join the memory pool.

enormous numbers of effectors capable of killing infected cells and releasing cytokines upon antigen encounter. Such a massive CD8⁺ T-cell response is not unique to LCMV infection, as large numbers of antigen-specific T cells are generated in other human and animal infections^{95–99}. In this case of a viral or bacterial pathogen in which multiple pathogen-derived epitopes are known and the specific CD8⁺ T-cell response to each one can be quantitated by tetramer staining, the composition of the memory pool reflects the epitope specificity of the effector population^{94,100}. The dominant epitopes in the initial response remain dominant in the memory repertoire.

The problem for the immune system is how to revert to homeostasis from a situation at the height of response when over half of the CD8⁺ T cells in the infected animal are effector cells specific for one pathogen. The system copes extremely well. During the few days after antigen has been cleared, 90–99% of the effector cells die, leaving an expanded population of new memory cells. The original naïve CD8⁺ T-cell pool is left intact, except for the promotion of the pathogen-specific members, whereas the memory pool adds some new members (Fig. 4). A recent experiment in which memory T cells were irreversibly marked by the activation of a modified granzyme B promoter suggests that a specific subset of effector cells contributes to memory¹⁰¹. What regulates the contraction of the overwhelming numbers of effector CD8⁺ T cells into reasonable numbers of memory cells is unknown. There is no evidence that Fas/FasL or any other tumour-necrosis factor family member is involved in the massive apoptosis of the effector cells¹⁰². Cessation of antigen stimulation and withdrawal of growth factors at the end of the response may lead to death. By whatever mechanism the immune system pulls it off, this represents a remarkable example of the recovery of homeostasis in the T-cell repertoire.

The lives of a T cell

CD4⁺ and CD8⁺ T cells depend on TCR interactions with self-peptides presented by MHC molecules for both their maturation in the thymus and their maintenance in peripheral lymphoid organs. The signals delivered in the periphery through interactions with self maintain the homeostasis of the naïve T-cell pool by keeping cells alive and by stimulating a low level of proliferation when the need arises. Low-affinity interactions with self-peptide–MHC may keep naïve T cells alert and is a way for them to reaffirm their potential to respond to higher affinity interactions with foreign peptide. The recognition of self-peptide–MHC by developing thymocytes does more than simply deliver a survival signal—it also allows the preferential selection of T cells on the basis of their ability to detect self-ligands with high specificity and low affinity. Because the composition of self-peptides bound to MHC is diverse, the specificity of T cells selected to mature is also diverse. The fact that peripheral T cells remain sensitive to self-peptide–MHC suggests that a continuum of self-recognition ensures diversity within the T-cell pool long after positive selection in the thymus is completed.

The factors that govern the production and maintenance of the memory T-cell pool are a mystery. This issue is further compounded by the finding that memory T cells are heterogeneous and can be subclassified into those that retain receptors for entering secondary lymphoid organs and those that preferentially enter inflamed tissues and perform immediate effector function¹⁰³. In contrast to naïve T cells, T cells that have responded to foreign antigen and graduated to the memory pool no longer require self-antigen stimulation to survive. An obvious rationale for this is that once a T cell has responded to a first encounter with a pathogen, it does not need to reaffirm its usefulness in the memory pool. A pathogen that came once may certainly come again. Another reason why memory T cells should be less responsive to self-antigen is that they are potentially more dangerous than naïve cells. Memory T cells are easier to trigger, faster to respond, and may enter tissues more readily than naïve T cells, allowing them to see a broader range of self-antigens. Thus, compared with naïve T cells, they pose an increased threat to

upset the balance of discrimination between self and non-self. On the basis of this realization, one may propose a way in which a fraction of effector cells from a primary response is allowed to revert to memory status. The effector cells that have responded well to foreign antigen, yet are the least sensitive to low-affinity self-peptide–MHC interactions, may be the ones chosen to enter the memory pool. Their lower sensitivity to self-antigen would guard against a harmful autoimmune response due to unforeseen cross-reactivity with self. This lowered sensitivity to self may be based on the specificity of their TCR in a polyclonal population, or on some stochastic change in their ability to perceive low-affinity ligands in a monoclonal setting.

The goal of vaccination is to induce long-lived memory cells. Understanding the developmental progression of T lymphocytes from the thymus, to naïve peripheral cell, to effector cell to memory cell is a challenge for the future. Advances in the way that we are able to identify T cells on the basis of their specificity, and expanding knowledge of signalling pathways will allow rapid progress in this area.

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Commitment to the B-lymphoid lineage depends on the transcription factor Pax5

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The *Pax5* gene encoding the B-cell-specific activator protein (BSAP) is expressed within the haematopoietic system exclusively in the B-lymphoid lineage, where it is required *in vivo* for progression beyond the pro-B-cell stage. However, Pax5 is not essential for *in vitro* propagation of pro-B cells in the presence of interleukin-7 and stromal cells. Here we show that pro-B cells lacking Pax5 are also incapable of *in vitro* B-cell differentiation unless Pax5 expression is restored by retroviral transduction. *Pax5*^{−/−} pro-B cells are not restricted in their lineage fate, as stimulation with appropriate cytokines induces them to differentiate into functional macrophages, osteoclasts, dendritic cells, granulocytes and natural killer cells. As expected for a clonogenic haematopoietic progenitor with lymphomyeloid developmental potential, the *Pax5*^{−/−} pro-B cell expresses genes of different lineage-affiliated programmes, and restoration of Pax5 activity represses this lineage-promiscuous transcription. Pax5 therefore plays an essential role in B-lineage commitment by suppressing alternative lineage choices.

All types of blood cell are generated from a pluripotent haematopoietic stem cell (HSC) through developmentally restricted progenitors which undergo lineage commitment and subsequent differentiation along a single pathway. The lymphoid lineages develop through a common lymphoid progenitor (CLP) which gives rise to natural killer, B and T cells¹. Early B-cell development can be dissected into different stages according to the rearrangement status of the immunoglobulin heavy-chain (*IgH*) locus, the expression of stage-specific cell-surface markers and growth factor requirements^{2,3}. The earliest B-lineage precursor cells carry the *IgH* locus still in germline configuration. *D_H-J_H* recombination is subsequently initiated in pre-BI cells³, which are also known as early pro-B cells (fraction B)². These pro-B cells can be cultured *in vitro* on stromal cells in the presence of interleukin-7 (IL-7) and express the B-cell surface proteins λ5, VpreB, Igα and Igβ (refs 3, 4). Completion of a functional *V_H-D_H* rearrangement results in the expression of the pre-B-cell receptor and subsequent differentiation to small pre-B cells, which are no longer responsive to pro-B-cell growth conditions⁵.

The initiation of B-cell development critically depends on two transcription factors; the basic helix–loop–helix proteins encoded by the *E2A* gene and the early B-cell factor (EBF). In the absence of either protein, B-cell development is aborted at the earliest stage, before *D_H-J_H* rearrangement of the *IgH* gene^{6–8}. Moreover, forced expression of E2A and EBF in haematopoietic precursor cells revealed that these regulators cooperatively induce the transcription of several B-lymphoid-specific genes^{9,10}. Hence, loss- and gain-of-function experiments have implicated E2A and EBF in the control of B-lineage commitment.

A third transcriptional regulator involved in early B-lymphopoiesis is the B-cell-specific activator protein (BSAP), which is encoded by the *Pax5* gene and recognizes DNA through the highly conserved paired domain characteristic of the Pax family of transcription factors¹¹. Within the haematopoietic system, *Pax5* is exclusively expressed in the B-lymphoid lineage from the earliest detectable precursor to the mature B-cell stage^{12,13}. In the absence of Pax5, B-cell development is arrested in the fetal liver at a similarly early stage as in *E2A* and *EBF* mutant mice, whereas it proceeds in adult bone marrow up to the early pro-B (pre-BI) cell stage^{14,15}. B-lymphopoiesis in the bone marrow is, however, not advanced by expression of a functionally rearranged μ-heavy-chain transgene⁵, indicating that

the observed developmental block does not result from the inability of *Pax5*^{−/−} pro-B cells to undergo *V_H-D_H* rearrangements at the *IgH* locus¹⁵. *Pax5*^{−/−} pro-B cells can be cultured *in vitro* with similar efficiency as wild-type pro-B cells, a fact that has facilitated the genetic identification of Pax5 target genes¹⁵. BSAP (Pax5) functions both as a transcriptional activator and as a repressor, as it positively controls *CD19*, *mb-1* (*Igα*), *N-myc* and *Lef-1* expression and negatively regulates *PD-1* transcription¹⁶. Apart from these target genes, Pax5-deficient and wild-type pro-B cells do not significantly differ in the expression of 50 B-cell-associated genes analysed¹⁶. This observation suggested that pro-B cells, even in the absence of Pax5, still undergo commitment to the B-lymphoid lineage. However, investigating the development potential of *Pax5*^{−/−} pro-B cells, we now show that Pax5, instead of E2A and EBF, is the critical transcription factor involved in B-lineage commitment.

Pax5^{−/−} pro-B cells lack B-lymphoid potential

The *RAG2* mutation also blocks B-cell development at the pro-B-cell stage *in vivo*¹⁷, like the *Pax5* mutation¹⁵, but it is compatible with

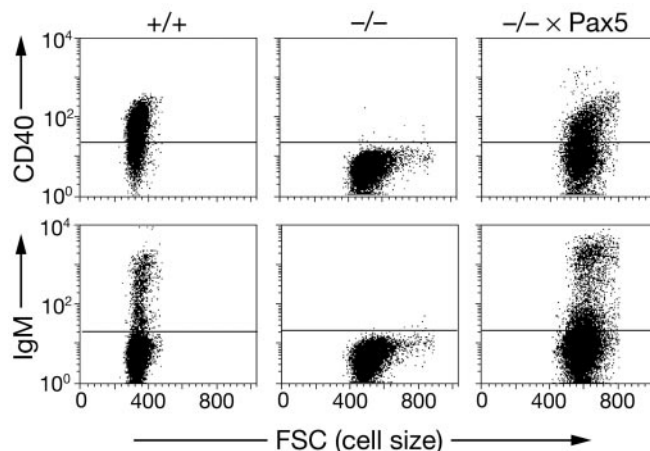


Figure 1 Pax5 is essential for *in vitro* differentiation of B lymphocytes. Wild-type and *Pax5*^{−/−} pro-B cells expressing a retroviral *bcl2* gene were cultured in the absence of stromal cells and IL-7 for three days in IMDM medium alone followed by flow cytometric analysis. Gating for CD19⁺ cells was used to select the *Pax5*^{−/−} pro-B cells infected with a BSAP (Pax5)-expressing retrovirus¹⁶. FSC, forward scatter.

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differentiation of these cells to mature B-lymphocytes *in vitro*¹⁸. We have therefore examined the *in vitro* differentiation potential of *Pax5*^{-/-} pro-B cells by culturing them for three days without IL-7 and stromal (ST2) cells. To promote cell survival upon IL-7 withdrawal¹⁸, a retroviral *bcl2* gene was introduced into *Pax5*^{-/-} and wild-type pro-B cells. In the absence of IL-7 and ST2 cells, the wild-type cells complete the rearrangement of their immunoglobulin genes and differentiate to small CD40⁺IgM⁺ B cells¹⁸ (Fig. 1). In contrast, *Pax5*^{-/-} cells do not express any markers associated with late differentiation (Fig. 1; and data not shown). However, *Pax5*^{-/-} pro-B cells infected with a *Pax5*-expressing retrovirus can differentiate into B cells *in vitro* (Fig. 1). Hence, the *Pax5* mutation arrests B-cell development at the same early stage *in vitro* as *in vivo*, and this developmental block can be overcome by retrovirus-mediated expression of *Pax5*.

Pax5^{-/-} pro-B cells are uncommitted

While growing pro-B-cell clones, we found that *Pax5*^{-/-} pro-B cells, in contrast to wild-type pro-B cells, could survive for up to three weeks without medium change. Although the culture medium was depleted of IL-7 during this time, some *Pax5*^{-/-} cells underwent a characteristic change in morphology and subsequently continued to proliferate. Whereas *Pax5*^{-/-} pro-B cells grown in IL-7 are small and refractile, and contain little cytoplasm (Fig. 2a), these cells became larger and irregular in shape upon IL-7 depletion (Fig. 2b).

To systematically investigate this phenomenon, we sorted individual pro-B cells into single wells of a 96-well plate, scored the growth of pro-B-cell colonies after 10 days in IL-7 medium and then monitored the frequency with which these colonies changed their morphology as IL-7 became limiting (Fig. 2c). For this purpose, pro-B cells were either freshly isolated from the bone marrow of *Pax5*^{-/-}, *RAG2*^{-/-} and wild-type mice (*ex vivo*) or were sorted from established pro-B cell cultures (*in vitro*). The *Pax5*^{-/-} pro-B-cell clones changed their morphology at a high frequency (64–87%), irrespective of *bcl2* expression, whereas the wild-type and *RAG2*^{-/-} pro-B cells did not show this morphology change (Fig. 2c). Our interpretation of these results is that the wild-type and *RAG2*^{-/-} pro-B cells have undergone B-lineage commitment and can therefore only differentiate to non-proliferating B-cells upon IL-7

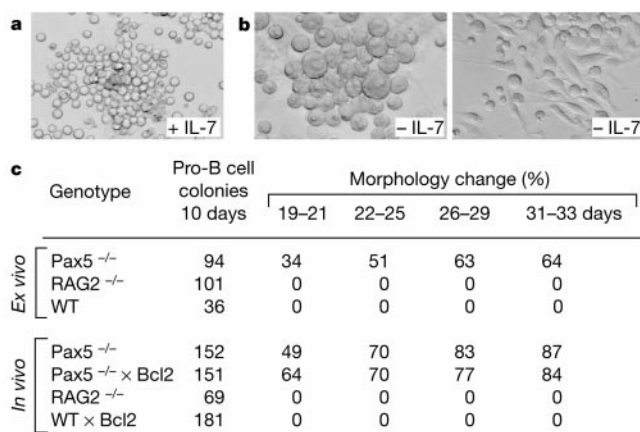


Figure 2 *Pax5*-deficient pro-B cells are not restricted to the B-lymphoid lineage.

a, Confocal microscopic image of a *Pax5*^{-/-} pro-B-cell colony after 10 days in IL-7 medium. **b**, Altered morphology of two representative colonies of the same pro-B-cell culture 30 days after the last medium change. **c**, Statistical analysis. Individual B220⁺ c-Kit⁺ pro-B cells, which were isolated by single-cell sorting directly from the bone marrow (*ex vivo*) or from established pro-B-cell cultures (*in vitro*) of the indicated genotypes, were grown on stromal ST2 cells in IL-7 medium, scored for colony formation at day 10 and subsequently examined for altered morphology at 3-day intervals (without medium change). The *bcl2* gene was introduced by retroviral transduction. WT, wild-type.

withdrawal¹⁸ (Fig. 1). However, under the same conditions *Pax5*^{-/-} pro-B cells cannot develop along the B-lymphoid lineage (Fig. 1). Instead, they appear to differentiate along other haematopoietic pathways, indicating that these cells are not committed to the B-lymphoid lineage.

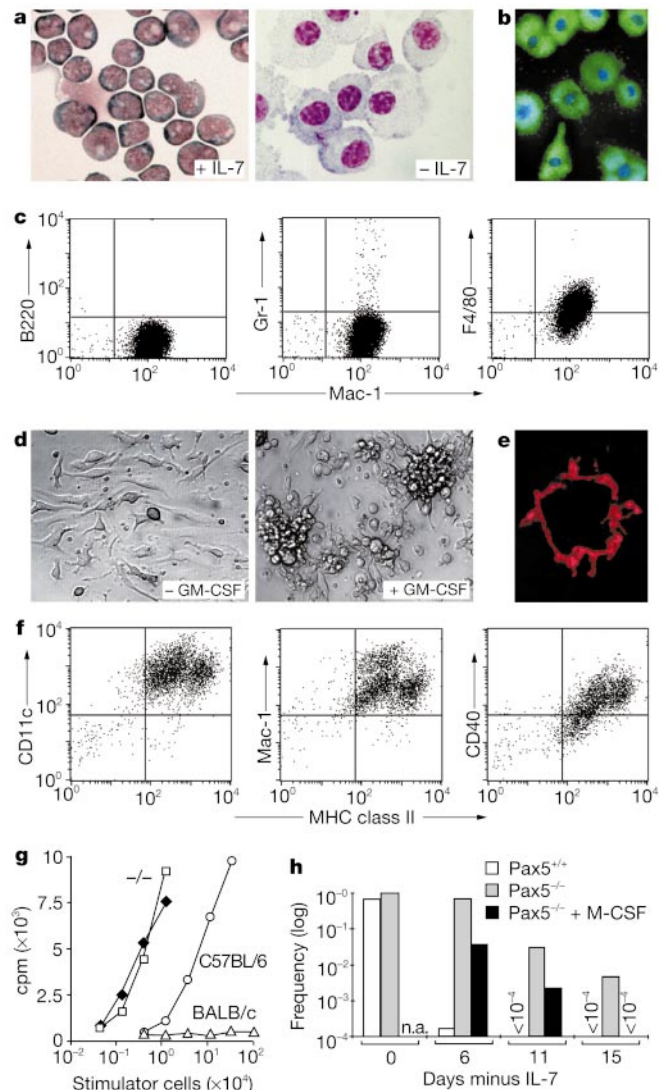


Figure 3 *In vitro* generation of macrophages and dendritic cells. **a**, May–Grünwald–Giemsa staining of cytoplasm preparations of *Pax5*^{-/-} pro-B cells cultured for 14 days on ST2 cells with or without IL-7. **b**, Phagocytosis. *In vitro* differentiated *Pax5*^{-/-} macrophages were incubated with FITC-labelled *E. coli*. **c**, Flow cytometric analysis of macrophages derived from *Pax5*^{-/-} pro-B cells. **d**, Confocal microscopic images of *Pax5*^{-/-} pro-B cells grown for 16 days on ST2 cells with or without GM-CSF. **e**, MHC class II expression on cells differentiated in GM-CSF medium and stained with an anti-MHC class II antibody. **f**, Flow cytometric analysis of dendritic cells derived from *Pax5*^{-/-} pro-B cells. The cells shown in **c** and **f** were gated on large size. **g**, Mixed leukocyte reaction. Increasing doses of γ -irradiated stimulator cells (cells per well) were incubated for four days with 2.5×10^5 responder cells isolated from lymph nodes of BALB/c mice. [³H]thymidine incorporation (c.p.m.) was measured during the last 14 h of incubation. Dendritic cells derived from two *Pax5*^{-/-} pro-B-cell lines (squares, diamonds) and splenocytes from allogeneic C57BL/6 (circles) or syngeneic BALB/c mice (triangles) were used as stimulator cells. **h**, Kinetic analysis of myeloid commitment. *Bcl2*-expressing pro-B cells deprived of IL-7 and ST2 cells were cultured with or without M-CSF. Viable cells were assessed for their ability to be re-cloned as pro-B cells in the presence of IL-7 and ST2 cells by limiting dilution analysis⁴. The re-cloning frequency is indicated relative to that of *Pax5*^{-/-} pro-B cells at day 0. n.a., not applicable.

In vitro differentiation along monocytic lineages

The morphology of the cells shown in Fig. 2b was suggestive of myeloid cell types, in agreement with the fact that ST2 cells produce the cytokine macrophage colony-stimulating factor (M-CSF)¹⁹. We therefore assessed the potential of *Pax5*^{-/-} pro-B cells to develop into macrophages by culturing them for 10–14 days on ST2 cells (in the absence of IL-7) followed by propagation in M-CSF (with or without ST2 cells). Cells differentiated in this manner displayed the characteristic morphology of enlarged vacuolar macrophages (Fig. 3a) and were positive for the macrophage markers Mac-1 and F4/80, but did not express the B-cell protein B220 and granulocyte marker Gr-1 (Fig. 3c). The same cells efficiently phagocytosed fluorescently labelled *Escherichia coli* (Fig. 3b), indicating that *Pax5*^{-/-} pro-B cells can differentiate into mature macrophages.

Using a cell-cloning assay, we next investigated the kinetics with which *Pax5*^{-/-} pro-B cells become committed to the myeloid lineage. *Bcl2*-expressing wild-type and *Pax5*^{-/-} pro-B cells were cultured in the absence of IL-7 and ST2 cells or in the presence of M-CSF for defined times. Subsequently, the proportion of viable cells that could be re-cloned as pro-B cells upon re-addition of stromal cells and IL-7 was determined by limiting dilution analysis. Wild-type pro-B cells rapidly lost their ability to be re-cloned (Fig. 3h) owing to efficient differentiation along the B-lymphoid lineage⁴. In contrast, the *Pax5*^{-/-} pro-B cells, which are not yet committed to a lineage-specific programme, could be re-cloned even after 15 days of IL-7 and stromal cell withdrawal (Fig. 3h). M-CSF addition, however, reduced the cloning frequency of *Pax5*^{-/-} pro-B cells 10-fold after six days and completely abolished it after 15 days (Fig. 3h). Hence, most cells initiated differentiation along the myeloid lineage and thus lost their IL-7 responsiveness within six days of M-CSF stimulation.

Dendritic cells also develop in the bone marrow from the same monocyte precursor as the macrophages, but require the cytokine granulocyte-macrophage CSF (GM-CSF) instead of M-CSF for terminal differentiation^{20,21}. To generate dendritic cells from *Pax5*^{-/-} pro-B cells, we applied a similar strategy as for macrophage development except that GM-CSF was added in the final differentiation step. After 16 days of GM-CSF stimulation, the cells were prolifer-

ating in small aggregates characteristic of dendritic cells²⁰, whereas only macrophages grew in the absence of GM-CSF (Fig. 3d). Mature dendritic cells are antigen-presenting cells characterized by a stellate shape with multiple cytoplasmic projections and by high levels of MHC class II protein on their surfaces^{20,21}. The *Pax5*^{-/-} cells, differentiated in response to GM-CSF, exhibited the characteristic cell morphology and MHC class II expression (Fig. 3e) as well as the expected cell-surface phenotype (MHC-II^{high} CD11c⁺ Mac-1⁺ CD40⁺ CD8⁺ B220⁻) of mature dendritic cells (Fig. 3f; and data not shown). These cells were further identified as dendritic cells by their ability to stimulate T-cell proliferation in a mixed leukocyte reaction (Fig. 3g). In this assay, allogeneic splenocytes of C57BL/6 mice elicit a proliferative response in T cells of BALB/c mice, in contrast to syngeneic splenocytes (Fig. 3g). Importantly, the *Pax5*^{-/-} dendritic cells activated the T cells with a 10–30-fold higher efficiency than allogeneic splenocytes (Fig. 3g), in agreement with the fact that dendritic cells are significantly more potent than splenocytes in stimulating T-cell proliferation²¹. *Pax5*^{-/-} pro-B cells can therefore also differentiate to become functional dendritic cells.

Osteoclasts constitute a third lineage which originates from a common monocyte precursor. Differentiation to bone-resorbing osteoclasts is controlled by the cytokine TRANCE (also known as OPGL, ODF or RANKL), which is presented on activated osteoblasts²². Following IL-7 withdrawal, we therefore cultured *Pax5*^{-/-} pro-B cells on stromal ST2 cells which ectopically expressed TRANCE. Under these conditions, the pro-B cells differentiated within two weeks into cells expressing the osteoclast-specific enzyme tartrate-resistant acid phosphatase (TRAP; Fig. 4a). These differentiated cells had the multinucleated morphology of mature osteoclasts (Fig. 4b) and formed resorption lacunae on artificial bone substrates (Fig. 4c). Hence, *Pax5*^{-/-} pro-B cells can also develop into functional osteoclasts.

Differentiation into granulocytes

Myeloid progenitors also give rise to cell types of the neutrophil lineage which characteristically contain a highly segmented nucleus and differentiate under the influence of the cytokines IL-6 and granulocyte CSF (G-CSF)²³. To test the granulocytic potential of *Pax5*^{-/-} pro-B cells, we cultured these cells for three weeks with the multilineage cytokines IL-3, IL-6 and stem-cell factor (SCF). Under these conditions, a small percentage of the *Pax5*^{-/-} cells differentiated into granulocytic cells, as shown by their segmented nuclear morphology (Fig. 4e). Homogeneous differentiation to granulocytes was achieved by replacing the multilineage cytokines with G-CSF for six days (Fig. 4f). *Pax5*^{-/-} pro-B cells can, therefore, also differentiate along the granulocytic lineage.

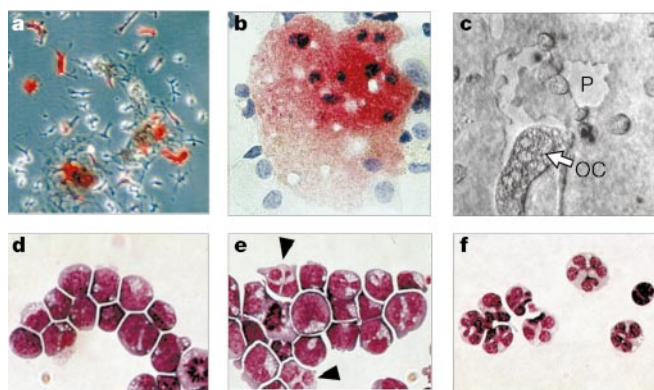


Figure 4 *In vitro* differentiation of osteoclasts and granulocytes. **a**, TRAP staining (red) of *Pax5*^{-/-} cells cultured for two weeks on TRANCE-expressing ST2 cells. **b**, Multinucleated morphology of TRAP⁺ cells. Differentiated *Pax5*^{-/-} cells were stained for TRAP activity (red) followed by counterstaining with haematoxylin (revealing the nuclei in blue) and cytopsin centrifugation. **c**, Bone resorption. Osteoclasts derived from *Pax5*^{-/-} pro-B cells were incubated on an osteologic bone disc with ST2-TRANCE cells. A multinucleated osteoclast (OC) is shown with the pits (P) resulting from its bone-resorbing activity. **d–f**, Cytopsin preparations of May-Grünwald-Giemsa-stained cells. *Pax5*^{-/-} pro-B cells initially grown in IL-7 medium (**d**) were cultured in the presence of IL-3, IL-6 and SCF for three weeks (**e**) and subsequently in G-CSF medium alone for six days (**f**). Granulocytes were identified by their segmented nuclear morphology (arrowhead in **e**).

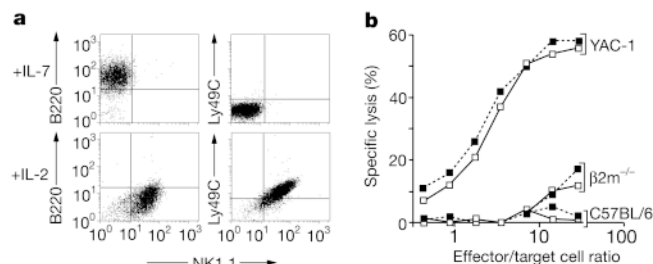


Figure 5 Generation of mature NK cells. **a**, Flow cytometric analysis of *Pax5*^{-/-} pro-B cells cultured for nine days with IL-2 and ST2 cells. **b**, Cytotoxicity. Cells of two *Pax5*^{-/-} pro-B-cell lines (closed and open symbols) were differentiated in IL-2 for nine days and assayed for their cytotoxicity against YAC-1 cells and LPS-treated splenocytes from wild-type C57BL/6 or $\beta 2m^{-/-}$ mice²⁷. Cytotoxicity assays were performed at the indicated effector to target cell ratios.

Differentiation into natural killer cells

The myeloid differentiation experiments unequivocally show that *Pax5*^{-/-} pro-B cells are not committed to the B-lymphoid lineage. Hence, the question arose of whether these cells also have properties of the recently described CLP and could thus give rise to natural killer (NK) cells¹. NK-cell development critically depends on IL-15 (ref. 24), which can, at least *in vitro*, be substituted by IL-2 (ref. 25). To assess their NK-cell potential, the *Pax5*^{-/-} pro-B cells were therefore cultured with IL-2 and stromal cells. Within 10 days, the *Pax5*^{-/-} cells switched off expression of the B-cell protein B220, initiated synthesis of the NK-cell markers NK1.1, Ly49C and 2B4 and were identified as NK cells by their cell-surface phenotype (NK1.1⁺ Ly49C⁺ 2B4⁺ IL-2Rα^{low} IL-2Rβ^{low} Mac-1^{low} CD3ε⁻ CD4⁻ CD8⁻ B220⁻; Fig. 5a and data not shown).

NK cells contribute to innate immunity by killing host cells that fail to express MHC class I proteins on their surfaces as a consequence of viral infection or tumorigenesis²⁶. We therefore measured the cytolytic activity of *in vitro* differentiated *Pax5*^{-/-} cells in a ⁵¹Cr release assay, using the sensitive YAC-1 tumour cells or LPS-stimulated lymphoblasts from β₂-microglobulin-deficient (β₂m^{-/-}) mice, which lack MHC class I cell-surface expression²⁷. Both target cells were lysed in a dose-dependent manner by NK cells derived from *Pax5*^{-/-} pro-B cells, whereas LPS-activated target cells from a syngeneic C57BL/6 mouse were resistant to lysis (Fig. 5b). Hence, *Pax5*^{-/-} pro-B cells can differentiate into functional NK cells.

Under all *in vitro* differentiation conditions analysed, we never detected *Pax5*^{-/-} cells that expressed surface antigens characteristic of the T-lymphoid lineage, in agreement with the fact that the complex inductive microenvironment of the thymus is required for early T-cell development. When injected into *RAG2*^{-/-} mice, *Pax5*-deficient pro-B cells were, however, able to fully reconstitute T-cell development *in vivo*²⁸. Therefore, *Pax5*^{-/-} pro-B cells can also give rise to lymphocytes except for B cells.

In vivo myeloid development of *Pax5*^{-/-} pro-B cells

The observation that injected *Pax5*^{-/-} pro-B cells home to the bone

marrow and undergo self-renewal in *RAG2*^{-/-} mice²⁸ raises the question of whether these cells can also differentiate *in vivo* into myeloid cells, in addition to T-lymphocytes. However, we could not detect the formation of myeloid cell types in reconstituted *RAG2*^{-/-} mice²⁸, suggesting that the *Pax5*^{-/-} pro-B cells cannot efficiently compete with endogenous myeloid progenitors. For this reason, we analysed the *in vivo* myeloid potential of *Pax5*^{-/-} pro-B cells in the *c-fos*^{-/-} mouse, which has a developmental defect in the osteoclast lineage^{29,30}. Consequently, *c-fos*^{-/-} mice develop osteopetrotic bones and, owing to the absence of a bone marrow cavity, exhibit extramedullary haematopoiesis in the spleen³⁰. As transplantation of *Pax5*^{-/-} pro-B cells alone cannot rescue lethally irradiated mice (data not shown), we performed competitive reconstitution experiments by injecting *Pax5*^{-/-} pro-B cells together with *c-fos*^{-/-} splenocytes into lethally irradiated, newborn *c-fos*^{-/-} mice. Four weeks after cell transfer, osteoclasts derived from *Pax5*^{-/-} pro-B cells could be detected by TRAP staining on the surface of the calvariae of three reconstituted *c-fos*^{-/-} mice (Fig. 6c), as in wild-type mice (Fig. 6a), whereas calvarial osteoclasts were never found in untreated *c-fos*^{-/-} mice (Fig. 6b). However, the reconstituted *c-fos*^{-/-} mice showed neither tooth eruption nor development of a bone marrow cavity in the long bones (Fig. 6f). A few clusters of TRAP⁺ osteoclasts were nevertheless observed in the osteopetrotic bones of the reconstituted *c-fos*^{-/-} mice (Fig. 6f), in contrast to untreated *c-fos*^{-/-} mice (Fig. 6e). Hence, the injected *Pax5*^{-/-} pro-B cells gave rise to only a partial rescue of the *c-fos* mutant phenotype, possible owing to still inefficient competition with myeloid progenitors derived from the *c-fos*^{-/-} HSC. Nevertheless, these experiments show that *Pax5*^{-/-} pro-B cells can differentiate into at least one myeloid cell type *in vivo*.

The multilineage potential is clonal

During this study, we tested the myeloid and lymphoid differentiation potential of five independently derived *Pax5*^{-/-} pro-B-cell pools, of which three were infected with a *bcl2*-expressing retrovirus. All five pro-B-cell lines developed into macrophages *in vitro* and T cells

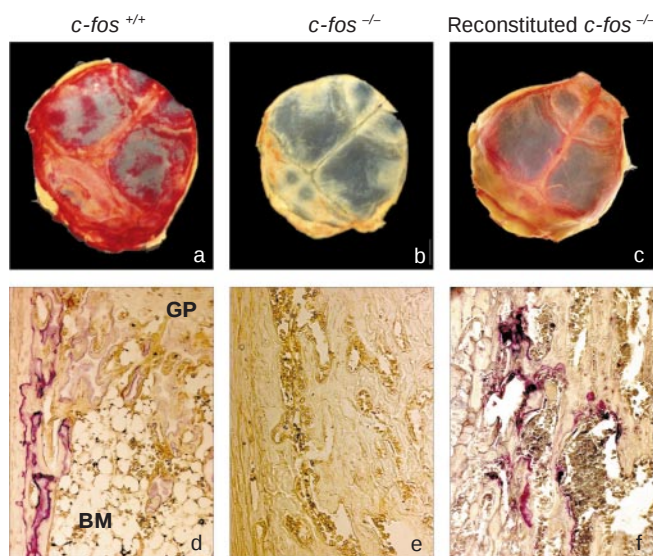


Figure 6 Development of osteoclasts from *Pax5*^{-/-} pro-B cells *in vivo*. Identification of osteoclasts in the calvariae (a–c) and long bones (d–f) of 4-week-old mice. TRAP staining (red) was used to detect osteoclasts on the calvarial surface and in sections of the tibia of wild-type (a, b), *c-fos*^{-/-} (b, e) and reconstituted *c-fos*^{-/-} (c, f) mice. For reconstitution, 10⁷ *Pax5*^{-/-} pro-B cells and 10⁷ splenocytes, isolated from an adult *c-fos*^{-/-} mouse, were co-injected intraperitoneally into lethally irradiated *c-fos*^{-/-} mice one day after birth. Four weeks later, osteoclast formation was analysed by TRAP histochemistry. BM, bone marrow cavity; GP, growth plate.

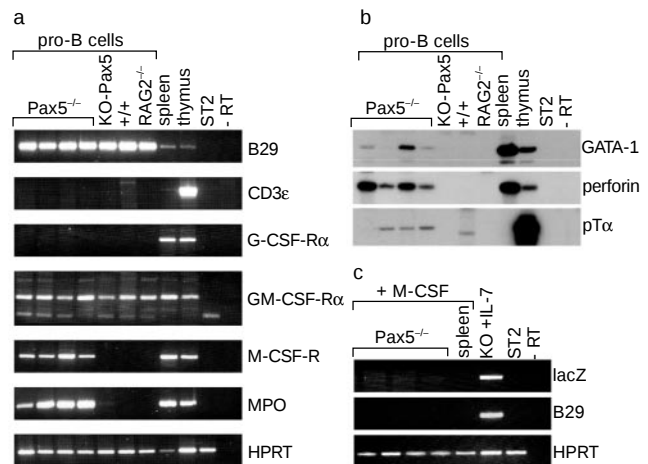


Figure 7 Lineage-promiscuous gene expression in *Pax5*^{-/-} pro-B cells. **a, b**, RT-PCR analysis. The following pro-B cells grown on stromal ST2 cells in the presence of IL-7 were analysed: four independent *Pax5*^{-/-} pro-B cell lines, *Pax5*^{-/-} pro-B cells reconstituted with a *Pax5*-expressing retrovirus (KO-*Pax5*) and pro-B cells established from wild-type (+/+) and *RAG2*^{-/-} bone marrow. The PCR products were visualized on agarose gels by ethidium bromide staining (a) or Southern blot analysis (b). The quantity of input cDNA was normalized by analysing the control *HPRT* transcript. No reverse transcriptase was added in lane – RT. **c**, Loss of *Pax5* expression upon myeloid differentiation. *Pax5*^{-/-} pro-B cells and wild-type splenocytes were cultured for 10 days with M-CSF and ST2 cells. *Pax5* expression was subsequently monitored by RT-PCR with primers from *Pax5* exon 1A and *lacZ* sequences inserted in the targeted *Pax5* locus¹⁴. *Pax5*^{-/-} pro-B cells grown in IL-7 medium (KO + IL-7) were used as positive control.

in vivo, indicating that the ability to differentiate along the myeloid and lymphoid lineages is an intrinsic property of all *Pax5*^{-/-} pro-B cells, irrespective of *bcl2* expression. However, it could be argued that, despite their growth in IL-7, the *Pax5*^{-/-} pro-B-cell pools may correspond to a heterogeneous mixture of lineage-committed progenitors. To exclude this possibility, we have analysed the differentiation potential of eight subclones which were generated by single-cell sorting of *Pax5*^{-/-} pro-B-cell pools. In the appropriate environment, all eight subclones efficiently developed into macrophages, osteoclasts, dendritic cells (*in vitro*) and T cells (*in vivo*). The differentiation into granulocytes and NK cells was variable, possibly indicating either that our *in vitro* differentiation conditions were not optimal for these two lineages or that *in vitro* cultured *Pax5*^{-/-} pro-B cells gradually lose the capacity to generate these two cell types. Nevertheless, our results unequivocally show that *Pax5*^{-/-} pro-B cells are multipotent haematopoietic progenitors which maintain their differentiation potential after single-cell cloning.

Pax5 represses lineage-promiscuous transcription

Multipotent haematopoietic progenitors simultaneously express genes from different lineage-affiliated programs, in a process known as 'multilineage priming'³¹. Consistent with its progenitor status, the *Pax5*^{-/-} pro-B cell is therefore expected to exhibit lineage-promiscuous gene expression, even though *Pax5*^{-/-} pro-B cells differ only minimally from committed wild-type pro-B cells in the expression of 50 B-cell-associated genes analysed¹⁶. To investigate this issue, we used the polymerase chain reaction with reverse transcription (RT-PCR) to compare the expression of different lineage-specific genes between uncommitted *Pax5*^{-/-} pro-B-cell lines and committed pro-B cells derived either by retrovirus-mediated reconstitution of *Pax5* expression or by isolation from wild-type and *RAG2*^{-/-} bone marrow (Fig. 7a, b). According to their expression pattern, the different genes could be grouped into three categories (Fig. 7; and data not shown). The transcripts of several genes, including *CD3ε* (T-lymphoid), *G-CSF-Rα* (granulocytic) and *β^{maj}-globin* (erythroid), were not detected in any pro-B-cell line, whereas they could be readily amplified from control tissues. Other genes were equally expressed in pro-B cells of all genotypes. This category comprises the genes *B29* (B-lymphoid), *GM-CSF-Rα* (myeloid), *EPO-R* (erythroid), *Tcf-1* (T-lymphoid) and *GATA-3* (T-lymphoid), although the last two transcription factor genes were expressed only at a very low level. Some genes, on the other hand, were exclusively expressed in *Pax5*^{-/-} pro-B cells, but not in any of the committed pro-B cells. This group includes the relatively abundant transcripts of the myeloid M-CSF receptor (*M-CSF-R*; *c-fms*) and myeloperoxidase (*MPO*) genes as well as the rare messenger RNAs of the *GATA-1* (erythroid), *perforin* (NK cell) and *pTα* (T lymphoid) genes. Importantly, the lineage-promiscuous expression of these genes was efficiently repressed in *Pax5*^{-/-} pro-B cells by restoring *Pax5* activity using retroviral transduction (Fig. 7a, b).

We next investigated whether the transcription of B-cell-specific genes such as *B29* (*Igβ*) and *Pax5* is repressed in *Pax5*^{-/-} pro-B cells upon entry into the myeloid lineage. Ten days after replacement of IL-7 with M-CSF, four independent *Pax5*^{-/-} cell lines efficiently downregulated the expression of *B29* as well as of the *lacZ* gene inserted in the targeted *Pax5* locus (Fig. 7c). In summary, our data indicate that an essential function of *Pax5* in B-lineage commitment is to repress the lineage-promiscuous transcription of non-B-lymphoid genes. Conversely, transcription of the *Pax5* gene is itself inactivated upon differentiation to other haematopoietic lineages.

Discussion

Differentiation of the HSC into distinct blood cell types is thought to progress through intermediate progenitor cells with restricted developmental potential. This view of haematopoiesis is challenged

by our finding that the *Pax5*^{-/-} pro-B cell possesses, at least under *in vitro* conditions, a broad developmental potential similar to that of the HSC itself. So far, however, we have been unable to differentiate the *Pax5*^{-/-} pro-B cell along the erythroid and megakaryocytic lineages, which may reflect a more restricted developmental capacity of this cell or our failure to define appropriate differentiation conditions for these lineages. Moreover, *Pax5*^{-/-} pro-B cells have so far failed to reconstitute the entire haematopoietic system in transplantation experiments²⁸. In the absence of this characteristic property of the HSC, the *Pax5*^{-/-} pro-B cell must be classified as a haematopoietic progenitor cell with broad lymphoid and myeloid differentiation potential.

A close developmental connection between the lymphoid and myeloid lineages has been demonstrated by the identification of a B-cell/macrophage-restricted precursor, on the basis of several criteria³². First, certain human acute leukaemias co-express B-cell and macrophage characteristics, indicating that they may be derived from a transformed progenitor cell of both lineages³³. Second, mice expressing the *Eμ-myc* transgene generate immature lymphomas that can differentiate along the B-lymphoid and macrophage lineages^{34,35}. Third, several immortalized pre-B-cell lines can be converted into macrophages³⁶⁻³⁸. Finally, the murine fetal liver contains a small subpopulation of clonogenic B-cell/macrophage progenitors³⁹. Fetal liver cells with an expanded lymphomyeloid potential have been identified by several groups by varying the conditions of their *in vitro* differentiation assays⁴⁰⁻⁴². A progenitor cell with a similar developmental capacity has so far not been isolated from mouse bone marrow¹, whereas human bone marrow contains cells with the potential to differentiate along the T, B, NK and dendritic cell lineages⁴³. The interpretation of these studies is, however, complicated by the fact that the isolated progenitor cells are rare, correspond to only a transition stage in haematopoietic development and cannot be expanded long-term *in vitro* in an uncommitted state, thus precluding any detailed analysis. In contrast, the *Pax5*^{-/-} pro-B cell can be propagated *in vitro* as an uncommitted haematopoietic progenitor. Moreover, this cell originates from the bone marrow and, except for B-cell development, exhibits the full spectrum of myeloid and lymphoid differentiation.

The development of lymphocytes, but not of myeloid cells, is critically dependent on IL-7, which promotes the proliferation and/or survival of lymphoid precursors⁴⁴. Expression of the IL-7 receptor was used as a criterion for isolating the CLP from mouse bone marrow¹. This CLP has the capacity to rapidly reconstitute B, T and NK cells *in vivo*, but lacks myeloid differentiation potential *in vivo* and *in vitro*¹. A close relationship between the CLP and *Pax5*^{-/-} pro-B cell is suggested by the fact that the *Pax5*^{-/-} pro-B cell also expresses the IL-7 receptor¹⁵, depends on IL-7 for *in vitro* propagation¹⁵ and possesses lymphoid developmental potential. However, in contrast to the CLP, the *Pax5*^{-/-} pro-B cell can differentiate along myeloid lineages *in vitro* and generate osteoclasts *in vivo* after injection into *c-fos*^{-/-} mice. Given the transitory nature of the CLP, it is conceivable that, shortly after its isolation, the CLP may express *Pax5* or an equivalent commitment factor of the NK- and T-cell lineages, which subsequently prevents differentiation along myeloid lineages. In this view, the full developmental capacity of the CLP is only revealed under conditions that interfere with commitment to the lymphoid lineages, such as IL-7-dependent proliferation in the absence of *Pax5* function.

Multilineage priming of gene expression is a characteristic property of early haematopoietic progenitor cells⁴⁵. This process ensures that genes of different haematopoietic lineages are co-expressed within a single cell, albeit often at a low level³¹. The lineage-promiscuous gene expression observed in the *Pax5*^{-/-} pro-B cell thus provides molecular evidence for the progenitor status of this cell. The expression of *M-CSF-R*, *GM-CSF-Rα* and *Epo-R* may also explain why the *Pax5*^{-/-} pro-B cell can respond to different

cytokines. For instance, expression of the M-CSF receptor renders the *Pax5*^{-/-} pro-B cell responsive to M-CSF, which is produced by the ST2 feeder cells used for pro-B-cell culture¹⁹. IL-7 is, however, dominant over M-CSF in lymphoid precursor cells³⁸. As a consequence, M-CSF can support monocytic differentiation and proliferation only at limiting IL-7 concentrations, which explains the observed morphology change of *Pax5*^{-/-} pro-B cells in IL-7-depleted medium.

Early events in lineage specification involve the control of proliferation, survival and commitment of lineage-restricted precursor cells. The loss of an entire lineage by gene targeting can therefore result from interference with any one of these processes and does not necessarily imply a role of the mutated gene in lineage commitment. A hallmark of commitment is the stabilization of a lineage-specific gene-expression programme which permanently inhibits alternative lineage choices⁴⁵. Consequently, restriction of developmental plasticity provides a better criterion for defining lineage commitment. Loss-of-function experiments can therefore implicate a transcription factor in lineage commitment only if the lack of 'forward' differentiation is accompanied by maintenance of developmental plasticity in the transcription-factor-deficient progenitor cell. However, it must be possible to grow a mutant progenitor *in vitro* before its developmental potential can be analysed. So far, *Pax5* is the only lineage-specific transcription factor to fulfil this criterion, as *Pax5*^{-/-} pro-B cells can differentiate along multiple myeloid and lymphoid lineages with the exception of the B-cell pathway. Restoration of *Pax5* activity overcomes the B-cell developmental block, thus identifying *Pax5* as the critical B-lineage commitment factor. Surprisingly, considerable progression down the B-cell pathway is possible in the absence of commitment, as *Pax5*^{-/-} pro-B cells express many genes previously considered to be indicative of B-lineage commitment^{15,16}. These genes include *Igα*(*mb-1*), *Igβ*(*B29*), *VpreB* and *λ5* as well as the sterile transcripts of the *IgH* locus, all of which are under single or combinatorial control by the transcription factors E2A and EBF^{7-9,46}. Consistently, both E2A and EBF, which act upstream of *Pax5* in the genetic hierarchy of B-cell development^{7,8}, are equally expressed in *Pax5*^{-/-} and wild-type pro-B cells¹⁵. Hence, the *Pax5* mutation dissociates the initial activation of B-lineage-specific gene expression by E2A and EBF from the *Pax5*-dependent control of B-lineage commitment. *Pax5* fulfils a dual role in B-lineage commitment, as it activates the expression of B-cell-specific genes and simultaneously represses the lineage-promiscuous transcription of other haematopoietic genes. The transcriptional activation is best exemplified by *CD19*, whose expression is strictly dependent on *Pax5* (ref. 16) and should thus be regarded as a decisive marker of B-lineage commitment. Moreover, the *Pax5*-dependent repression of the *M-CSF-R* gene illustrates at the molecular level how the developmental potential is restricted at commitment by rendering B-cell precursors unresponsive to lineage-inappropriate cytokines such as M-CSF.

A long-standing debate concerns whether lineage commitment in the haematopoietic system occurs in a cell-autonomous, stochastic manner or is controlled by instructive signals from the local environment^{47,48}. Much attention has been paid to the role of haematopoietic growth factors in this process, but no evidence for any defect in lineage commitment has been observed despite the analysis of many growth factor gene knock-outs⁴⁸. Instead, growth factors seem to perform a selective role by promoting the survival and/or proliferation of independently committed cells. We have shown that the expression of *Pax5* is randomly initiated from only one of its two alleles at the onset of B-lymphopoiesis⁴⁹. The transcription of a single allele indicates that the lineage commitment gene *Pax5* is activated in a relatively inefficient, stochastic manner in the multipotent progenitor⁴⁹. However, once this stochastic event has occurred, it restricts the various developmental options of the progenitor to the B-cell pathway. Hence, our data

support the notion that B-lineage commitment is a stochastic rather than a deterministic process. □

Methods

Mice

Pax5^{-/-}, *RAG2*^{-/-} and *c-fos*^{-/-} mice^{14,17,29} were maintained on the C57BL/6x129/Sv background and genotyped as described^{5,15,29}.

Pro-B cell culture

Iscoe's modified Dulbecco's medium supplemented with 50 μM β-mercaptoethanol, 1 mM glutamine, 2% heat-inactivated fetal calf serum and 0.03% (w/v) primatone was used for all cell-culture experiments (referred to as IMDM medium). Pro-B cells isolated from bone marrow of 2-week-old mice were cultured on γ-irradiated ST2 cells in IMDM medium containing IL-7 (ref. 15).

Retroviral infection

The retrovirus pBabe-BSAP has been described¹⁶; pBabe-TRANCE was provided by K. Matsuo and E. F. Wagner. Murine *bcl2* cDNA was inserted into the histidinol resistance vector pMV-10 to generate a *bcl2*-expressing retrovirus. Transfected GP + E-86 packaging cells were selected with 2.5 μg ml⁻¹ puromycin or 10 mM histidinol (Sigma). Pro-B cells were infected by coculture with puromycin-histidinol-resistant ST2 cells and virus-producing packaging cells followed by puromycin (2.5 μg ml⁻¹) or histidinol (1 mM) selection.

Antibodies

Anti-CD40 (FGK45.5), anti-μH (M41), anti-CD19 (1D3), anti-c-Kit (ACK4) and anti-MHC class II (M5-114) antibodies were purified and biotinylated as described¹⁵. Fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-labelled anti-B220 (RA3-6B2), FITC-labelled anti-Ly49C (5E6) and anti-Mac-1 (M1/70), PE-conjugated CD11c (HL3) and Gr-1 (RB6-8C5), biotinylated anti-CD23 (B3B4) and anti-NK1.1 (PK136) antibodies and allophycocyanin (APC)-conjugated streptavidin were obtained from Pharmingen; PE-conjugated anti-F4/80 (C1:A3-1) antibody from Serotec; and PE-labelled streptavidin from Southern Biotechnology Associates.

Flow cytometry

Cells were stained and analysed on a FACSCalibur (Becton Dickinson) as described¹⁴. Biotinylated antibodies were revealed with PE- or APC-streptavidin. Myeloid cells were incubated with 10% heat-inactivated rat serum before antibody staining.

Growth factors

Murine M-CSF (used at a final concentration of 25 ng ml⁻¹), G-CSF (25 ng ml⁻¹) and SCF (100 ng ml⁻¹) were purchased from R&D Systems. All other cytokines were produced by X63 or J558L cell transfectants⁵⁰ and used at a concentration of 1% (IL-7), 2% (IL-3, IL-6), 5% (IL-2, IL-4) and 10% (GM-CSF) conditioned supernatant.

Cell morphology assay

Individual B220⁺c-Kit⁺ pro-B cells from bone marrow (*ex vivo*) or long-term pro-B-cell cultures (*in vitro*) were sorted by a FACS Vantage TSO flow cytometer (Becton Dickinson) into single wells of 96-well plates containing ST2 cells in IL-7 medium. Colonies of small, round, refractile pro-B cells were identified after 10 days and thereafter scored for morphology changes defined by an increase in cell size, elongation and acquisition of granular vacuolated cytoplasm.

Cell-cloning assay

The cloning frequency of pro-B cells cultured on ST2 cells plus IL-7, in medium alone or in the presence of M-CSF, was assessed by limiting dilution analysis as described⁴. Cell numbers were normalized for viable cells (determined by trypan blue exclusion) before dilution into 96-well plates.

In vitro differentiation

All differentiation assays were performed with *in vitro* cultured pro-B cells. B lymphocytes: pro-B cells were incubated at 1–3 × 10⁶ cells per ml for three days in IMDM medium alone as described¹⁸. NK cells: pro-B cells were differentiated for 10 days in the presence of IL-2 and ST2 cells. Cytotoxicity assays were performed with YAC-1 cells or LPS-stimulated splenocytes as described²⁵. Macrophages: *Pax5*^{-/-} pro-B cells were differentiated in IMDM medium with ST2 cells for 10–14 days followed by terminal differentiation in M-CSF medium for seven days. Cells were transferred to chamber slides (Nalge Nunc), incubated with 10⁷ FITC-labelled heat-inactivated *E. coli* (Molecular Probes) for 1–2 h at 37 °C, washed twice in PBS, fixed in 2% paraformaldehyde for 10 min, air-dried, mounted in VectaShield (Vector Laboratories) and DABCO (10 μg μl⁻¹, Sigma)–DAPI (0.15 μg μl⁻¹, Sigma) solutions (1:1) and analysed on a fluorescence microscope (Zeiss Axiophot) with a CCD camera (Photometrics). Dendritic cells: *Pax5*^{-/-} pro-B cells were differentiated for 16 days on ST2 cells in GM-CSF medium. For immunocytochemical analysis, cells were flushed off the stromal cell layer and stained with PE-anti-MHC class II antibodies before cytospin centrifugation and fluorescence microscopy. Differentiated cells were tested for antigen presentation by the mixed leukocyte reaction as described²⁰. Osteoclasts: *Pax5*^{-/-}

pro-B cells were cultured for two weeks with M-CSF and ST2 cells expressing TRANCE. Cells were fixed in 3.7% formaldehyde for 30 min, dried and subjected to TRAP staining (Sigma). At day 10 of differentiation, cells were transferred onto osteologic bone discs (Millenium Biologic) together with ST2–TRANCE cells in M-CSF medium containing 100 nM dexamethasone, 10 mM vitamin D₃ and 50 µg ml⁻¹ vitamin C. Bone resorption was analysed after an additional 10-day incubation. Granulocytes: *Pax5*^{-/-} pro-B cells were cultured for three weeks in IMDM medium containing IL-3, IL-6 and SCF and then incubated for six days in IMDM medium containing G-CSF alone. The cell morphology was analysed by May–Grünwald–Giesma staining.

In vivo osteoclast development

Newborn mice were lethally irradiated (800 rad) and then intraperitoneally injected with a mixture of 10⁷ *in vitro* cultured *Pax5*^{-/-} pro-B cells and 10⁷ splenocytes from an adult *c-fos*^{-/-} mouse. Four weeks later, osteoclast formation was analysed by TRAP staining of isolated calvariae and histological sections of long bones³⁰.

RT-PCR

Total RNA (2 µg) from pro-B cells and lymphoid tissues was reverse-transcribed and PCR-amplified as described⁴⁹. The following primers were used: *Pax5-lacZ*, 5'-CATGGCGA-GAAGCTCTTTAGTTCC-3' and 5'-TGCAAGGCGATTAAAGTTGGGTAAC-3', *CD3ε*, 5'-GTCTCCATCTCAGGAACCACT-3' and 5'-ATAGTCTGGGTTGGGAACAGG-3'. All other primers have been described^{15,25,28,31}. PCR products were identified on agarose gels by ethidium bromide staining or Southern hybridization.

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ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28

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The T-cell-specific cell-surface receptors CD28 and CTLA-4 are important regulators of the immune system. CD28 potently enhances those T-cell functions that are essential for an effective antigen-specific immune response^{1–5}, and the homologous CTLA-4 counterbalances the CD28-mediated signals and thus prevents an

otherwise fatal overstimulation of the lymphoid system^{6–9}. Here we report the identification of a third member of this family of molecules, inducible co-stimulator (ICOS), which is a homodimeric protein of relative molecular mass 55,000–60,000 (M_r 55K–60K). Matching CD28 in potency, ICOS enhances all basic T-cell responses to a foreign antigen, namely proliferation, secretion of lymphokines, upregulation of molecules that mediate cell–cell interaction, and effective help for antibody secretion by B cells. Unlike the constitutively expressed CD28, ICOS has to be *de novo* induced on the T-cell surface, does not upregulate the production of interleukin-2, but superinduces the synthesis of interleukin-10, a B-cell-differentiation factor. *In vivo*, ICOS is highly expressed on tonsillar T cells, which are closely associated with B cells in the apical light zone of germinal centres, the site of terminal B-cell maturation. Our results indicate that ICOS is another major regulator of the adaptive immune system.

We identified ICOS by generating monoclonal antibodies against activated human T cells. The ICOS-specific monoclonal antibody F44 did not react with resting human peripheral-blood T cells, but stained CD4⁺ and CD8⁺ T lymphocytes that had been activated by stimulation of the T-cell antigen receptor (TCR) complex (Fig. 1a). No signal was detected on resting or appropriately activated B cells, monocytes, natural killer cells, granulocytes, dendritic cells or platelets (data not shown). Immunoprecipitations using monoclonal

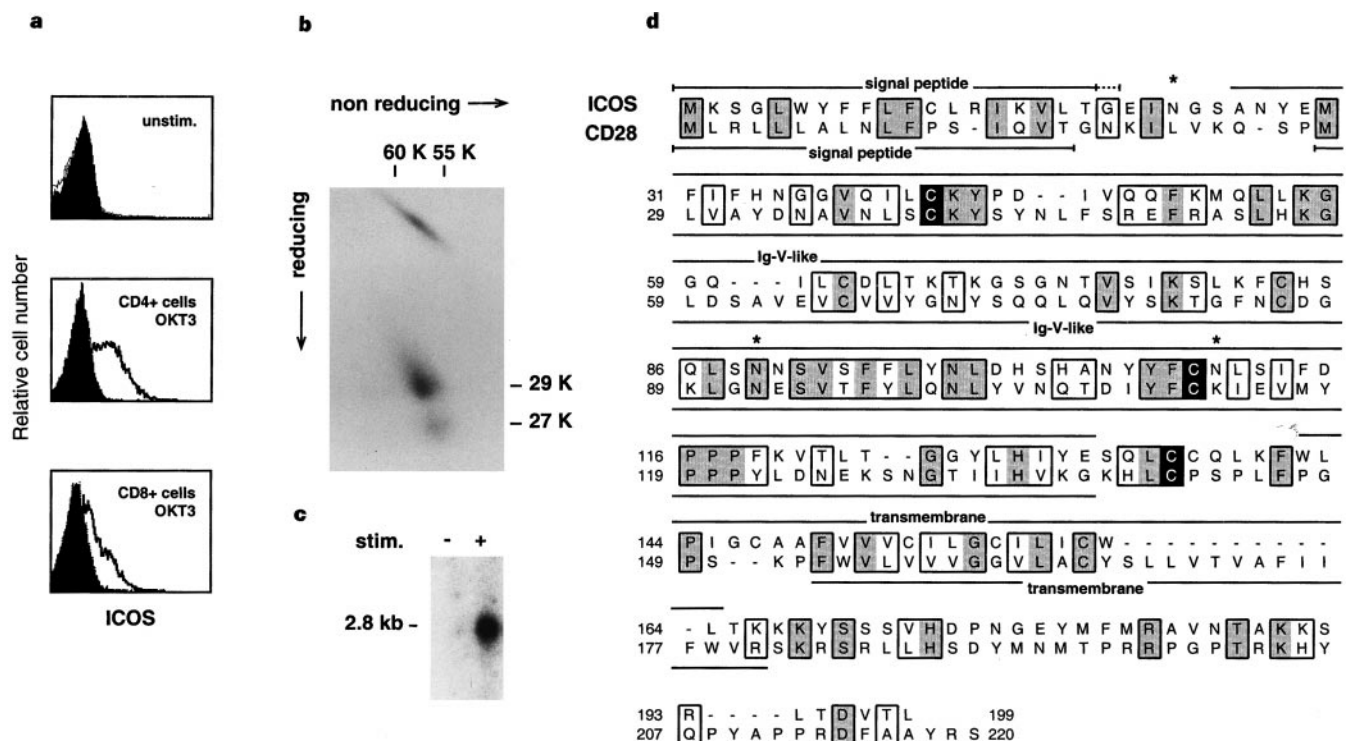


Figure 1 Identification, purification and cloning of ICOS. **a**, Expression of ICOS on human T cells after 36 h of stimulation. Peripheral-blood CD4⁺ or CD8⁺ T cells were left untreated or were stimulated with the solid-phase-bound anti-CD3 monoclonal antibody OKT 3 (1:1,000 dilution of ascites), and were analysed by flow cytometry using the fluorescein isothiocyanate (FITC)-labelled monoclonal antibody F44. At 14 h after stimulation, ICOS could not yet be detected on CD8⁺ T cells, whereas CD4⁺ T cells expressed levels of ICOS that were similar to those expressed after 36 h (data not shown). The background signal obtained with the isotype-control monoclonal antibody MOPC-21 (Sigma) is shown in black. Stimulation by phorbol-12-myristate-13-acetate (PMA) and ionomycin led to the expression of ICOS on most CD4⁺ and CD8⁺ T cells (data not shown). **b**, Structure of the homodimeric ICOS protein. ICOS protein was immunoprecipitated from surface-iodinated MOLT-4V cells with monoclonal antibody F44 and separated by two-dimensional (non-reducing/reducing) SDS–PAGE. Numbers at the top and right side of the gel are M_r values. The 55K–60K protein species on the diagonal corresponds to residual dimeric

ICOS, which was not reduced by the in-gel reducing procedure required for the two-dimensional SDS–PAGE (a full reduction of this species was routinely observed in one-dimensional SDS–PAGE; data not shown). Identical data were obtained with iodinated activated primary T cells (data not shown). **c**, ICOS mRNA expression. Amounts of ICOS mRNA were determined by northern blot analysis of total RNA (2.5 µg per lane) obtained from human peripheral-blood CD4⁺ T cells activated with PMA (20 ng ml^{–1}) and ionomycin (200 ng ml^{–1}) for 24 h. **d**, Amino-acid-sequence alignment of ICOS and CD28 (ref. 28), obtained using the Clustal-W algorithm with BLOSUM 30 matrix (MacVector, Oxford Molecular Group). Identical amino-acid residues are shaded; conserved residues are boxed; potential *N*-glycosylation sites are indicated by asterisks. Predicted signal peptides (the exact cleavage site for ICOS has not been determined experimentally), IgV-like domains²⁹ and predicted transmembrane regions are indicated. Cysteine residues that may be important in maintaining three-dimensional structure are shown in black.

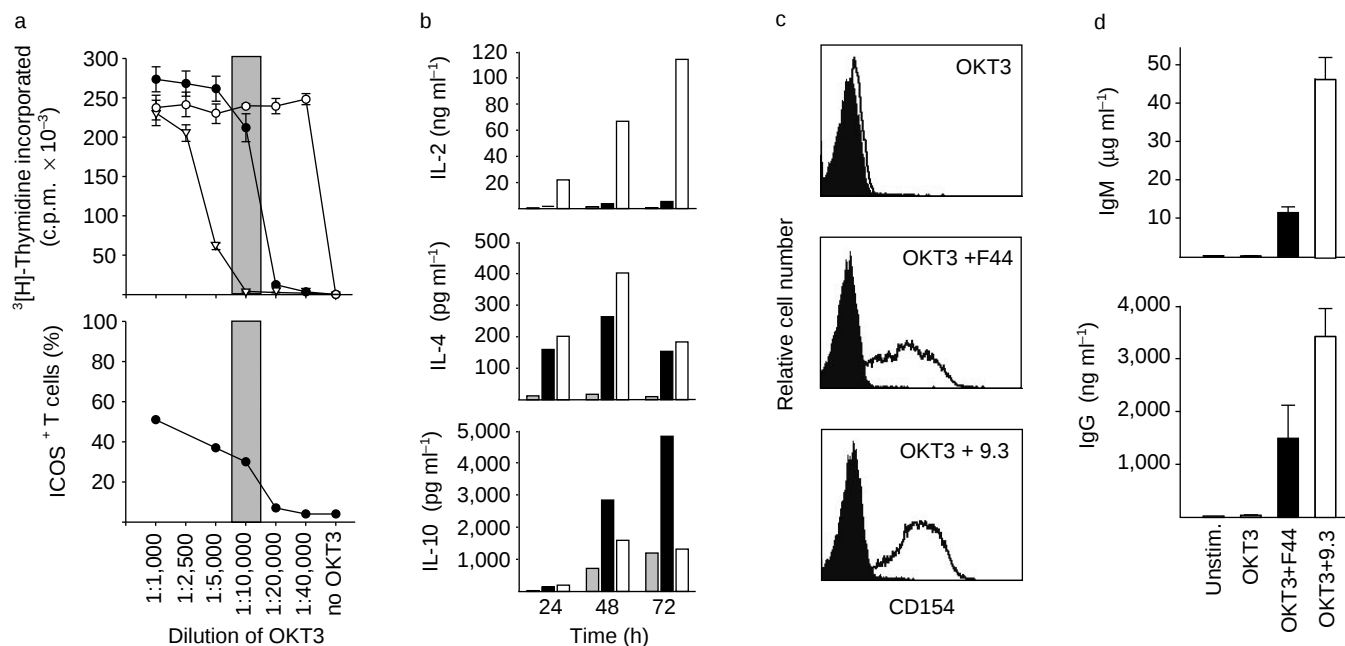


Figure 2 Co-stimulatory functions of ICOS. **a**, Co-stimulation of T-cell proliferation. Peripheral-blood CD4 $^{+}$ T cells were cultured in the presence of monoclonal antibody OKT 3 (dilutions of ascites), and monoclonal antibodies 9.3 (open circles), F44 (filled circles), or isotype-control MOPC-21 (triangles) (see Methods). Top, proliferation of T cells was determined by addition of 1 μCi [^3H]thymidine for the last 16 h of the 72 h culture period. The shaded area shows the experimental point at which maximal co-stimulation with F44 was observed. In ten experiments, co-stimulation through ICOS enhanced proliferation of T cells by 60- to 80-fold (range 14- to 135-fold) over the background stimulation observed with OKT 3 alone. Bottom, the percentage of T cells induced to express ICOS at the various dilutions of OKT 3 ascites in cultures with co-crosslinked F44 at 24 h after stimulation, the time point of maximal ICOS expression. **b**, Co-stimulation of lymphokine secretion by primary T cells. Peripheral-blood CD4 $^{+}$ cells were stimulated by optimal CD3-crosslinking (1:1,000 dilution of OKT 3 ascites) in the presence of co-stimulating monoclonal antibody 9.3 (white columns) or F44 (black columns), or in the presence of the control monoclonal

antibody MOPC-21 (stippled columns). Supernatants were collected at the indicated times after stimulation and assayed for various cytokines using enzyme-linked immunosorbent assays (ELISAs) (Biosource). Results of a representative experiment out of six are shown. **c**, Upregulation of the cell-surface molecule CD154 (also known as CD40 ligand and TRAP). Peripheral blood CD4 $^{+}$ T cells were suboptimally stimulated (1:5,000 dilution of OKT 3 ascites) in the presence of control monoclonal antibody MOPC-21 or monoclonal antibodies F44 or 9.3, and CD154 expression was determined by flow cytometry with an FITC-labelled antibody, TRAP1 (ref. 15). **d**, Effect of T-cell co-stimulation through ICOS on immunoglobulin synthesis by tonsillar B cells. Peripheral-blood CD4 $^{+}$ T cells were cultured with tonsillar B cells in microtitre plates precoated with monoclonal antibody OKT 3 (1:5,000 dilution of ascites) and MOPC-21, F44 or 9.3. IgM and IgG levels in the culture supernatants were determined by ELISA on day 8. Error bars indicate the s.d. from quadruplicate cultures. Results from a representative experiment out of six are shown.

antibody F44 defined the ICOS antigen as a disulphide-linked dimer, with an apparent relative molecular mass of 55K–60K, composed of a 27K and a 29K chain (Fig. 1b). We enriched ICOS protein from lysates of the ICOS-expressing T-cell line MOLT-4V by large-scale affinity chromatography using F44, and further purified the protein by two-dimensional preparative SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 1b). The 27K and 29K ICOS-protein species yielded identical peptide sequences, indicating that ICOS may be expressed on the cell surface as a homodimeric protein, with the two chains differing only in their post-translational modification; this assumption was later confirmed by transfection experiments (data not shown).

Using the peptide sequence, we cloned full-length ICOS complementary DNA (2,641 base pairs) from a MOLT-4V cDNA library. Northern analysis revealed a single ICOS messenger RNA species of ~ 2.8 kilobases in length in activated human T cells (Fig. 1c). The open reading frame of ICOS mRNA encodes a new protein of 199 amino acids with a predicted relative molecular mass of 22.6K. The ICOS amino-acid sequence shares 24% (17%) identity and 39% (39%) similarity with CD28 (Fig. 1d) (and CTLA-4 (ref. 10)). The predicted mature ICOS is a type-I transmembrane molecule (type I transmembrane proteins have their carboxy termini in the cytosol) that consists of a single immunoglobulin (Ig)V-like domain (stabilized by conserved cysteine residues at positions 42 and 109; Fig. 1d), a transmembrane region of ~ 23 amino acids, and a cytoplasmic tail of 35 amino acids, and it shows a close structural resemblance to CD28 (Fig. 1d) and CTLA-4 (ref. 10). The cysteine residue located at

position 141 of CD28, also found in CTLA-4, is apparently involved in forming the disulphide bridge between the homodimeric chains of these proteins, and is also found in ICOS (position 136). The motif MYPPPY (positions 117–122 in CD28), required in its intact form for the binding of CD28 and CTLA-4 to their counter-receptors B7-1 and B7-2 (refs 11, 12), is not conserved in ICOS, indicating that ICOS may interact with a different receptor.

In a series of *in vitro* experiments, we compared ICOS to the main co-stimulatory effects of CD28 directly. We tested the ICOS-specific monoclonal antibody F44 in parallel with monoclonal antibody 9.3, one of the most potent CD28-specific reagents available 1,13 . When human peripheral-blood CD4 $^{+}$ T cells were suboptimally activated through the CD3 complex of the TCR, co-stimulation of T-cell proliferation by ICOS was comparable in potency to signalling by CD28 (shaded area in upper panel of Fig. 2a). This finding was remarkable, given that ICOS was present on at most 30–40% of the CD4 $^{+}$ T cells under these experimental conditions (shaded area in lower panel of Fig. 2a), whereas CD28 continued to be expressed on nearly all CD4 $^{+}$ T cells. At higher dilutions of the CD3-specific monoclonal antibody OKT 3, the co-stimulatory effect of monoclonal antibody F44 could no longer be achieved, because the signal through the TCR complex was insufficient to induce the expression of the ICOS molecule on the T-cell surface (Fig. 2a, lower panel).

Only a small amount of most lymphokines is secreted from primary CD4 $^{+}$ T cells after optimal triggering of the CD3/TCR complex alone 14 . One characteristic of co-stimulation of CD4 $^{+}$ T cells by CD28 is the enhancement of cytokine secretion, in

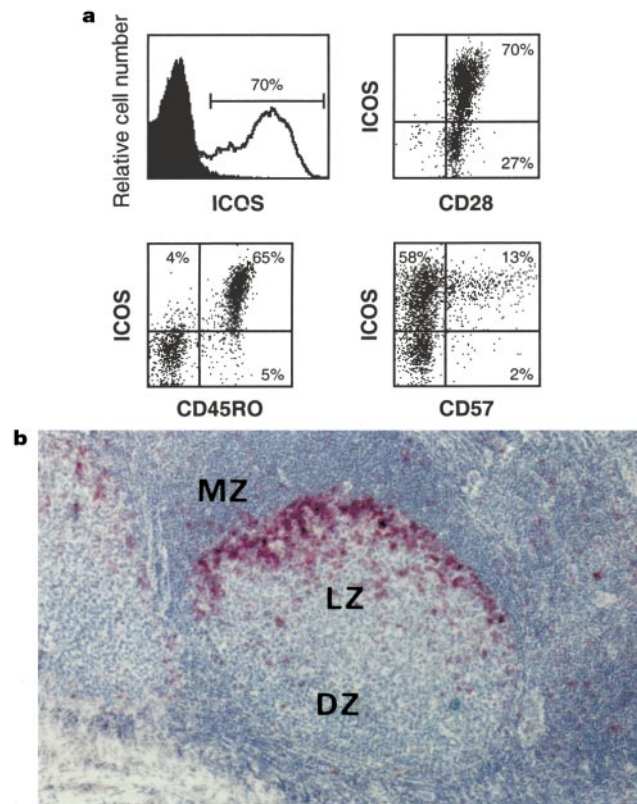


Figure 3 Expression of ICOS on tonsillar T cells. **a**, Tonsillar T lymphocytes were analysed by three-colour flow cytometry on a FACSCalibur (Becton Dickinson). T cells (5×10^4) were gated with a CD3-specific monoclonal antibody (UCHT1-CyChrome, Pharmingen) and expression of ICOS (monoclonal antibody F44–phycoerythrin) was correlated with the expression of CD28 (CD28.2–FITC), CD45RO (UCHL1–FITC), or CD57 (NC1–FITC; all

monoclonal antibodies from Immunotech). Data shown are representative of material obtained from 20 tonsils. **b**, Localization of ICOS⁺ cells in the apical light zone of germinal centres, as determined from histochemical stains of frozen human tonsillar sections with monoclonal antibody F44 using the APAAP technique³⁰. LZ, light zone; DZ, dark zone; MZ, mantle zone (original magnification $\times 69$).

particular the 'superinduction' of interleukin (IL)-2 (Fig. 2b and refs 1, 3, 4, 14). Surprisingly, co-stimulation by ICOS failed to significantly upregulate IL-2 at any time point (Fig. 2b). However, co-stimulation by ICOS upregulated the secretion of IL-4, IL-5, interferon- γ , tumour-necrosis factor- α and granulocyte/macrophage-colony-stimulating factor to 50–70% of the levels achieved with co-stimulation by CD28 (Fig. 2b and data not shown). ICOS and CD28 had markedly different co-stimulatory effects on the secretion of IL-10. In contrast to the modest stimulatory capacity of CD28, co-stimulation by ICOS routinely induced several-fold higher secretion of IL-10 (Fig. 2b); northern blot analysis confirmed the upregulation of expression of IL-10 (data not shown). As the proportion of ICOS⁺ T cells in the lymphokine-co-stimulation system did not exceed 50% (Fig. 2a, lower panel), on a per-cell basis ICOS matched the ability of CD28 to amplify the secretion of many lymphokines (except for IL-2), and was superior to CD28 in co-inducing IL-10.

T cells communicate with other cells of the immune system by *de novo* expression of cell-surface molecules, as well as by generating lymphokines. Co-stimulation of T cells through ICOS or CD28 markedly upregulated expression of CD154 (also known as CD40 ligand or TRAP), a molecule required for the crosstalk of T cells with B cells¹⁵ (Fig. 2c), and also other early T-cell-activation antigens such as CD69, CD25 or CD71 (data not shown). Next, we tested whether the ICOS-induced expression of a variety of lymphokines and cell-surface receptors was sufficient for the complex communication of T cells with tonsillar B lymphocytes. Unstimulated T cells, or T cells that had only been preactivated through the TCR, could not induce immunoglobulin synthesis by B cells (Fig. 2d). Only co-stimulation through ICOS or CD28 enabled T cells to

provide effective help for B cells to synthesize IgM and IgG (Fig. 2d). On a per-cell basis, co-stimulation through ICOS was as effective as co-stimulation through CD28 in upregulating the expression of cell-surface receptors by T cells or IgG synthesis by B cells.

To determine the function of ICOS *in vivo*, we stained tissue sections of various lymphoid organs with monoclonal antibody F44. In the adult thymus and spleen, ICOS expression was almost completely confined to the few small germinal centres that are present in these tissues (data not shown). In tonsils, a substantial number of cells stained positively for ICOS and we therefore characterized tonsillar cells extensively by flow cytometry. Of these tonsillar T cells, 50–70% expressed high levels of ICOS (Fig. 3a); all other tonsillar cell populations did not express ICOS (data not shown). The expression of ICOS was associated with the presence of CD28 on the T-cell surface (Fig. 3a). Nearly all ICOS⁺ T cells also expressed CD45RO (Fig. 3a), indicating that T cells bearing high levels of ICOS may be in a late phase of activation; ICOS⁻ T cells had the phenotype of resting cells (data not shown). Finally, almost all T cells positive for CD57, a marker for a subpopulation of T cells that are located in germinal centres of lymphoid tissues^{16,17}, co-expressed ICOS (Fig. 3a). Detailed immunohistological studies showed that ICOS is predominantly expressed on germinal-centre T cells located in the apical part of the germinal-centre light zone (Fig. 3b), a site in which T cells are known to induce terminal differentiation of B cells into antibody-secreting plasma cells or memory cells^{16,17}. We also identified a smaller but significant proportion of ICOS⁺ cells in the T-cell zone surrounding germinal centres (Fig. 3b).

On the basis of its structure, its T-cell-restricted expression and its function, we conclude that ICOS is closely related to CD28, an

important molecule in the immune system^{2–5,16,17}. However, despite their overall similarity, ICOS and CD28 differ profoundly in several aspects. In humans, CD28 is constitutively expressed at high levels on almost all CD4⁺ and on 50% of CD8⁺ T cells, whereas expression of ICOS must be induced *de novo*. Both CD28 and ICOS co-induce the synthesis of several lymphokines, but only CD28 superinduces IL-2, whereas ICOS is more effective in superinducing IL-10. It is likely that the interaction of CD28 with its counter-receptors B7-1 and B7-2 is important in the early phases of cooperation between T cells and B cells. At later time points, CTLA-4 counteracts the signals provided by CD28 (refs 6, 9, 18, 19) and thus terminates the production of IL-2; IL-2 is known to drive the expansion of the germinal-centre B cells^{17,20,21}. ICOS, probably binding to receptor(s) on B cells other than B7-1 and B7-2 (see above), may become the dominant signal for T cells in the later phases of T-cell–B-cell cooperation in the lymphoid system. Such a scenario is probable, because IL-10 can induce the terminal differentiation of B cells into memory cells and antibody-secreting plasma cells^{22–24}; this takes place in the apical light zone of the germinal centre^{16,17}, the major site of ICOS expression. □

Methods

Cell preparation, T-cell activation and generation of monoclonal antibodies

Peripheral-blood CD4⁺ (96% pure) and CD8⁺ (92% pure) cells were negatively purified from buffy coats using nylon wool adherence and magnetobead-coupled (Dynal) monoclonal antibodies specific for CD19, CD11b, major histocompatibility complex (MHC) II molecules, and CD8 or CD4. Peripheral-blood CD4⁺ or CD8⁺ T cells (1×10^5 in 200 μ l) were activated using the anti-CD3 monoclonal antibody OKT 3 (ATCC; ascites diluted as indicated in Fig. 2a), and were co-stimulated with monoclonal antibody 9.3 (anti-CD28; 1:3,000 dilution of ascites), F44 (anti-ICOS; 4 μ g ml⁻¹), or MOPC-21 (IgG1 isotype control; 4 μ g ml⁻¹) all of these antibodies were solid-phase-bound through precoating of the 96-well round-bottomed microtitre plates with rabbit anti-mouse serum (10 μ g ml⁻¹, Sigma).

To assay cooperation between T and B cells, peripheral-blood CD4⁺ T cells (50,000 per well) treated with mitomycin C were co-cultured with tonsillar B cells (25,000 per well) in 96-well round-bottomed microtitre plates. Tonsils, obtained from individuals (aged 3–18 yr) undergoing tonsillectomy, were mechanically dispersed and T cells (80–90% pure) were isolated using Ficoll–Hypaque gradient centrifugation followed by nylon-wool adherence. Tonsillar B cells were isolated by rosetting tonsillar cells with sheep red blood cells that had been treated with 2-aminoethyl isothiourrea.

Monoclonal antibodies were generated by fusing spleen cells of BALB/c mice that had been immunized with activated human T cells to myeloma P3X63Ag8.653 (ATCC). The hybridoma secreting the ICOS-specific monoclonal antibody F44 (IgG1) was obtained by subcloning of hybridoma 8F4.

Protein purification and sequencing

MOLT-4V cells (a variant of MOLT-4; ATCC) were lysed in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1% Nonidet P-40 (NP-40) at 4°C for 1 h (1 ml buffer per 20×10^6 cells). Nuclei and insoluble material were removed by centrifugation at 1,000g and 100,000g, respectively. The lysate was preabsorbed with unspecific monoclonal antibody coupled to CnBr-activated Sepharose (Pharmacia) and incubated at 4°C for 4 h with monoclonal antibody F44 coupled to protein G–Sepharose (Pharmacia) according to ref. 25. The F44 matrix was washed in a column with several volumes of buffer 1 (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM EDTA, 1 mM PMSF and 0.5% NP-40), buffer 2 (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.5% NP-40 and 0.1% SDS), and buffer 3 (0.2 M glycine, pH 4.0, and 0.5% CHAPS), and the bound protein was eluted with two volumes of 0.2 M glycine, pH 2.5, and 0.5% CHAPS. The eluate was concentrated by ultrafiltration (Centricon 10, Amicon) and subjected to preparative two-dimensional non-reducing/reducing gel electrophoresis (20×10^9 cell equivalents per gel). After electrophoresis in the first dimension, the gel area containing the ICOS protein was cut out and subjected to reducing conditions in 5.3 M urea, 0.5 M Tris-HCl, pH 8.0, 1% SDS, 1% β -mercaptoethanol (50°C for 1 h). Free cysteines were subsequently alkylated by addition of 10 mM iodoacetamide (37°C for 30 min). After equilibration in 1x SDS–PAGE sample buffer (30 min), the gel piece was mounted onto a SDS–PAGE slab gel (12% polyacrylamide gel). Following electrophoresis, Coomassie-blue-stained spots corresponding to the ICOS protein (Fig. 2b) were cut out. Protein material from five gels was pooled, *in situ*-digested with trypsin²⁶, fractionated by reverse-phase HPLC on a microRPC C2/C18 SC column (Smart system, Pharmacia) using a linear gradient of acetonitrile and 0.1% trifluoroacetic acid, and sequenced on a pulsed-liquid gas-phase sequencer (Applied Biosystems).

cDNA cloning

Poly(A)⁺ MOLT-4V RNA was converted to cDNA using oligo-dT primers and Superscript II reverse transcriptase (Gibco), and the cDNA was cloned into λ ZAPII vector (Stratagene) according to the manufacturer's instructions. Peptide XRLTDVT, obtained from protein

microsequencing, was used to derive the two degenerate oligonucleotides MGNCTSACNGAYGTNAC (512 permutations) and MGNYTACNGAYGTNAC (1,024 permutations) for screening of the generated cDNA library by hybridization in 3 M tetramethyl ammonium chloride²⁷. Several positive clones were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). One of the cDNA clones encoded the above peptide sequence (XRLTDVT) and was used to isolate a full-length cDNA clone.

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Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen

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Cytotoxic T lymphocytes (CTLs) are thought to detect viral infections by monitoring the surface of all cells for the presence of viral peptides bound to major histocompatibility complex (MHC) class I molecules. In most cells, peptides presented by MHC class I molecules are derived exclusively from proteins synthesized by the antigen-bearing cells¹. Macrophages and dendritic cells also have an alternative MHC class I pathway that can present peptides derived from extracellular antigens; however, the physiological role of this process is unclear². Here we show that virally infected non-haematopoietic cells are unable to stimulate primary CTL-mediated immunity directly. Instead, bone-marrow-derived cells are required as antigen-presenting cells (APCs) to initiate anti-viral CTL responses. In these APCs, the alternative (exogenous) MHC class I pathway is the obligatory mechanism for the initiation of CTL responses to viruses that infect only non-haematopoietic cells.

The 'classical' MHC class I antigen-presentation pathway is thought to be the major mechanism used by the immune system to detect viral infections in all cells. In this pathway, proteins synthesized by a cell are degraded in the cytoplasm into oligopeptides, a fraction of which are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP). In the ER these peptides bind to new MHC class I molecules and the resulting complexes are transported to the cell surface. As MHC class I molecules must bind peptides in order to be transported to the plasma membrane, TAP is required for normal MHC class I expression at the cell surface and for antigen presentation¹.

To determine whether non-haematopoietic cells can function as APCs to initiate CTL responses to viruses, we constructed bone-marrow chimaeras by lethally irradiating C57Bl/6 mice (B6 mice; MHC class I haplotype H-2^b) and reconstituting them with bone marrow from TAP^{0/0} mice³ (also H-2^b; all bone-marrow chimaeras will be referred to as bone-marrow donor → irradiated recipient). As bone-marrow-derived cells in TAP^{0/0} → B6 mice cannot transfer peptides from the cytosol to the ER, they are unable to use the classical MHC class I pathway^{3,4}.

The chimaeric mice were assayed for the generation of CTL responses following infection with wild-type vaccinia virus or with vaccinia-OVA, a recombinant vaccinia virus carrying chicken ovalbumin (OVA) as a full-length protein⁵. Although they have intact MHC class I antigen presentation in non-haematopoietic tissues, TAP^{0/0} → B6 mice did not generate CTL responses to vaccinia-viral antigens (Fig. 1a) or to OVA (Fig. 1b). In contrast, robust CTL responses to these antigens were detected in control B6 → B6 mice. These results indicate that the generation of CTL responses to vaccinia virus requires bone-marrow-derived cells with functional TAP molecules.

Next, we determined whether the inability of TAP^{0/0} → B6 mice to generate CTL responses was due to a defect in CD8⁺ T cells or to a failure in antigen presentation by bone-marrow-derived APCs. TAP^{0/0} (non-chimaeric) mice almost completely lack CD8⁺ T cells

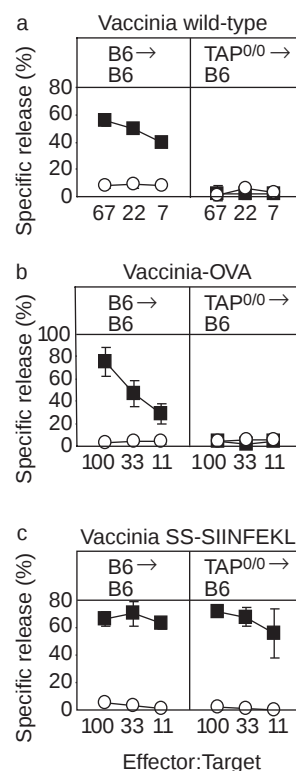


Figure 1 CTL responses to wild-type vaccinia virus and OVA in recombinant vaccinia virus requires a bone-marrow-derived APC. **a**, CTL response to wild-type vaccinia. The indicated bone-marrow chimaeras were infected with 2×10^7 p.f.u. of wild-type vaccinia virus. One week later, mice were killed and freshly explanted spleen cells were assayed in ⁵¹Cr-release assays on vaccinia-infected MC57G cells (filled squares) or uninfected MC57G cells as controls (open circles). The TAP^{0/0} → B6 mice did not generate a response to the vaccinia-viral antigens. **b**, CTL response to OVA in vaccinia-OVA protein. The indicated bone-marrow chimaeras were infected with 2×10^7 p.f.u. of vaccinia-OVA. One week later, mice were killed and their spleen cells were cultured in the presence of mitomycin-C (Sigma)-treated EG7 cells²⁹ (an EL-4-derived cell line stably transfected with OVA). After five days cells were collected and tested in ⁵¹Cr-release assays on EG7 targets (filled squares) on EL-4 targets as controls (open circles). **c**, CTL response to vaccinia-SS-SIINFEKL. The experiment was performed as in **b** except that mice were infected with vaccinia-SS-SIINFEKL. The x-axis shows the ratio of effector cells to target cells.

because expression of MHC class I molecules in epithelial thymic cells is necessary for the positive selection of CD8⁺ T cells in the thymus³. However, as shown by flow-cytometry analysis, TAP^{0/0} → B6 mice had normal numbers of CD8⁺ T cells in peripheral blood and spleen (ref. 4 and data not shown) as a consequence of positive selection on wild-type thymic epithelial cells (which are radioresistant and non-haematopoietic). These CD8⁺ T cells were fully functional. TAP^{0/0} → B6 mice and control B6 → B6 mice generated a similar CTL response to vaccinia-SS-SIINFEKL⁵ (Fig. 1c), a recombinant vaccinia virus expressing the antigenic peptide of OVA (SIINFEKL)⁶ preceded by a signal sequence that delivers the peptide directly into the ER, thus bypassing the need for TAP. TAP^{0/0} → B6 chimaeras and control mice also generated anti-SIINFEKL CTL when injected intravenously with a graded number of B6 dendritic cells that had been preincubated with a single concentration of synthetic SIINFEKL (not shown) or when injected with 5×10^5 dendritic cells that had been incubated with graded concentrations of SIINFEKL (Fig. 2). In these latter experiments, cells incubated with 10 nM SIINFEKL and cells infected *in vitro* with vaccinia-OVA and polio-OVA (see below) stimulated a T-cell

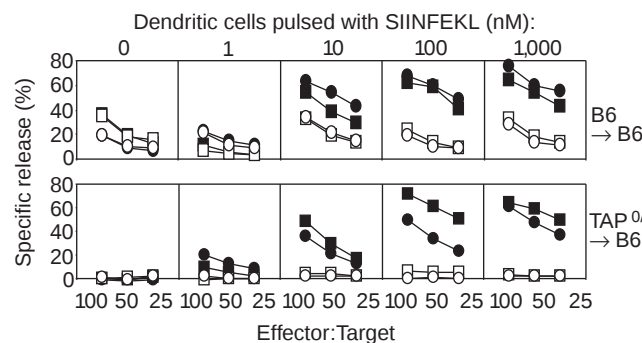


Figure 2 $TAP^{0/0} \rightarrow B6$ mice can generate CTL responses comparable to those in $B6 \rightarrow B6$ mice when immunized with APCs loaded with antigenic peptide. The indicated chimaeric mice were immunized intravenously with 5×10^5 B6-derived, *in vitro*-cultured dendritic cells that had been incubated with SIINFEKL peptide at the indicated concentrations. One week later mice were killed and their spleen cells restimulated *in vitro* for 4 days with mitomycin-C-treated EL-4 cells that had been incubated with SIINFEKL. For each panel, squares represent one mouse and circles represent another. Targets were EL-4 cells that had been preincubated with SIINFEKL (filled symbols) or EL-4 cells that had not been incubated with peptide as controls (open symbols).

hybrid specific for the peptide–MHC complex SIINFEKL–K^b at roughly similar levels (data not shown).

These results indicate that the inability of $TAP^{0/0} \rightarrow B6$ mice to generate a CTL response to vaccinia virus is due to a failure of antigen presentation. This failure to present antigens did not result from an inability of the $TAP^{0/0} \rightarrow B6$ chimaeras to reconstitute bone-marrow-derived APCs, because these mice generated MHC class II-restricted T-cell responses to OVA that were at least as strong as those generated by $B6 \rightarrow B6$ animals (Fig. 3a, b). Together these data show that bone-marrow-derived professional APCs, possessing a functional TAP, are required to initiate CTL responses to vaccinia, and that non-haematopoietic tissues infected with vaccinia cannot prime CTLs, despite the ability of vaccinia to infect many different tissues, including respiratory organs, liver, kidney, spleen, ovaries and the central nervous system^{7–9}. There may be several explanations for the inability of non-haematopoietic cells to stimulate a CTL response. Although non-haematopoietic cells are able to present antigenic peptides bound to MHC class I, they express low levels of these molecules in the absence of inflammation. Moreover, they do not express MHC class II molecules, which are essential for the stimulation of CD4⁺ helper T cells, and they lack adhesion and co-stimulatory molecules that might be required to stimulate naive T cells. Non-immune cells also lack the ability to migrate to lymphoid organs, where many immune responses are initiated¹⁰. On the other hand, some bone-marrow-derived cells, such as macrophages and dendritic cells, express high levels of MHC class I and class II molecules and several adhesion and co-stimulatory molecules, including B7.1 and B7.2, and can migrate to central lymphoid organs. However, after naive CTLs are stimulated to become effectors, they no longer require co-stimulation or T-cell help and can recognize lower levels of peptide–MHC complexes. Therefore, once stimulated by professional APCs, the effector CTLs acquire the ability to migrate out of the lymphoid organs to clear viral infections in all tissues.

In the experimental model described above, bone-marrow-derived APCs might acquire the viral antigens by becoming infected themselves¹¹. In fact, SIINFEKL was presented by vaccinia-OVA infected dendritic cells and macrophages *in vitro* (not shown). However, it seems unlikely that professional APCs would become infected by all viruses and therefore this mechanism would be unavailable to detect infections by many tissue-specific viruses. Alternatively, the bone-marrow-derived APCs might acquire vaccinia-viral antigens exogenously from other antigen-bearing

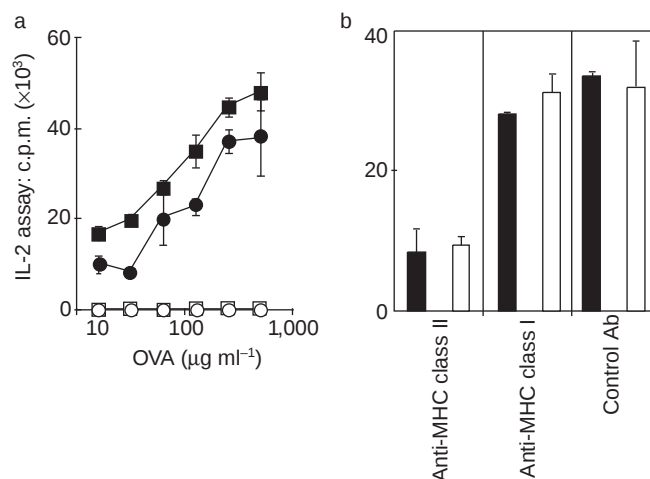


Figure 3 $TAP^{0/0} \rightarrow B6$ mice can generate MHC class II-restricted responses comparable to those in $B6 \rightarrow B6$ mice. **a**, Production of IL-2 by lymph-node cells of immunized chimaeras in response to different concentrations of OVA. Cells were from OVA-immunized $TAP^{0/0} \rightarrow B6$ mice (filled squares) and $B6 \rightarrow B6$ mice (filled circles) or from unimmunized controls (open squares and open circles respectively). **b**, The same cells as those used in **a** were incubated with 0.5 mg ml⁻¹ OVA in the presence of the indicated antibody-containing supernatants. Only the results for immunized $TAP^{0/0} \rightarrow B6$ mice (filled columns) or $B6 \rightarrow B6$ mice (open columns) are shown.

cells and this mechanism could operate in all infections, as proposed previously^{2,12}. To determine whether the presentation of exogenous antigen is important in viral immunity, we developed a model in which bone-marrow-derived cells could not be infected with a virus.

Poliovirus (polio) is a positive-strand RNA virus with a host range that includes humans but not mice. This host-range restriction is determined by the expression of a suitable poliovirus receptor (PVR) on host cells¹³. Cells from wild-type mice can not be infected with polio, but mouse cells transfected with human PVR can be infected (ref. 14 and data not shown). In this study we used two human PVR-transgenic mice. We constructed a transgenic mouse (cPVR, in an ICR background) expressing a full-length PVR complementary DNA, driven by the β -actin promoter, in all tissues studied. Such mice are susceptible to polio infection and die with poliomyelitis following injection with wild-type polio (not shown). Following intraperitoneal infection of cPVR and control (B6) mice with the wild-type strain of polio, we found much higher titres of virus (expressed as plaque-forming units per tissue) in the skeletal muscle (12,000), brain (1,600) and spinal cord (19,000), and slightly higher titres in the kidney (1.3) and liver (0.64), of the cPVR mice (B6-mouse titres: 0.70, <0.60, <0.30, <0.10 and <0.20, respectively). We mated cPVR mice with B6 mice, and their progeny, (cPVR $\times$ B6) F₁ mice (referred to here as cPVR mice), were used here. Another transgenic mouse strain (referred to here as gPVR) possesses the human PVR genomic locus, including the endogenous human promoter, backcrossed onto the B6 background. It also supports viral replication in skeletal muscle and central nervous system^{13,15}.

To examine the role of the exogenous MHC class I pathway in the initiation of CTL responses, we generated a series of bone-marrow-chimaeric mice, including two sets ($B6 \rightarrow$ cPVR and $B6 \rightarrow$ gPVR) that allowed infection of only non-bone-marrow-derived cells. In a previous study¹⁴, we constructed a polio virus recombinant (polio-OVA) expressing the carboxy-terminal half of OVA (which includes the SIINFEKL epitope). In this construct, the OVA fragment is synthesized as part of the viral polypeptide and is released in the cytosol by viral proteinases. We showed that polio-OVA can induce

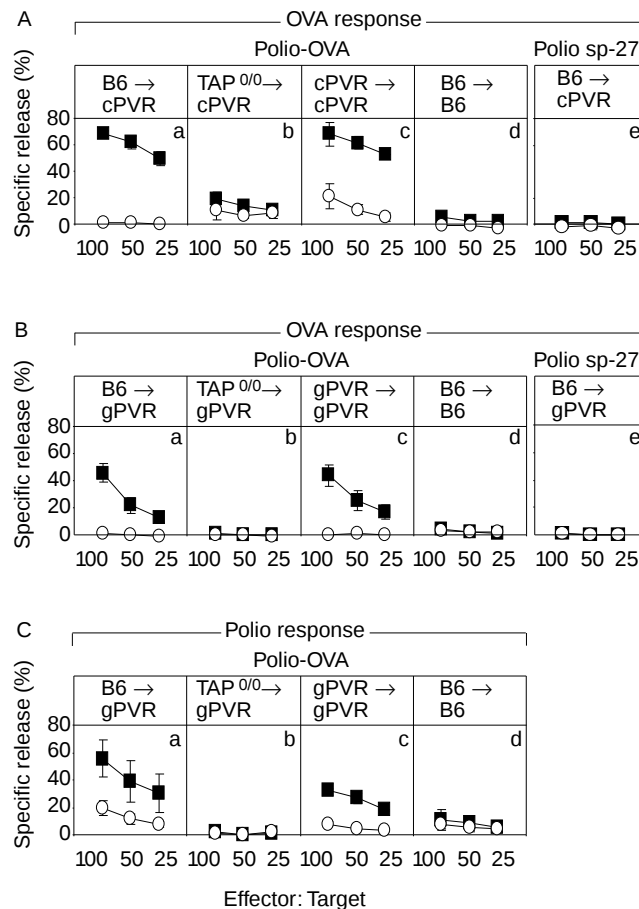


Figure 4 Initiation of the CTL response to polio-OVA requires the presence of bone-marrow-derived APCs, but not their infection. Each panel corresponds to a single experiment. **A, B**, Bone-marrow chimaeras (**Aa–e**, cPVR recipients and B6 control recipients; **Ba–e**, gPVR recipients and B6 control recipients) were infected with polio-OVA or polio-sp27 as indicated. Mice were killed 3 weeks later and their spleen cells were co-cultured for 5 days with mitomycin-C-treated EG7 cells and used in ^{51}Cr -release assays. Filled squares, EG7 targets; open circles, EL-4 targets as controls. **Ca–d**, Bone-

marrow chimaeras obtained using gPVR recipients and B6 control recipients were infected with polio-OVA. Three weeks later, mice were killed and their spleen cells were cultured in the presence of mitomycin-C-treated EL-4 cells that had been preincubated with $2\text{ }\mu\text{g ml}^{-1}$ of the D^b -binding polio-derived peptide P22. After 5 days, cells were collected and tested in ^{51}Cr -release assays. Targets were EL-4 cells preincubated with P22 (filled squares) or EL-4 cells without peptide as controls (open circles).

anti-OVA CTL responses in gPVR mice and cPVR but not in B6 mice (ref. 14 and data not shown). Consistent with this result, cPVR → cPVR mice infected with polio-OVA generated anti-OVA CTLs (Fig. 4Ac), but B6 → B6 mice did not (Fig. 4Ad). B6 → cPVR chimaeric mice, which have bone-marrow-derived cells that cannot be infected by polio (PVR-negative cells), generated strong anti-OVA CTL responses when infected with polio-OVA (Fig. 4Aa) but not when infected with a recombinant polio (polio-sp27) expressing an irrelevant protein (Fig. 4Ae). Identical results were obtained when using gPVR recipients (Fig. 4Ba, c and e). Therefore, either the infected non-haematopoietic cells are stimulating CTL responses, or bone-marrow-derived cells are acquiring the polio-expressed OVA from exogenous sources.

To distinguish between these two possibilities, we constructed chimaeric mice by using TAP^{0/0} mice as bone-marrow donors and PVR⁺ transgenic mice as recipients. Remarkably, TAP^{0/0} → cPVR (Fig. 4Ab) and TAP^{0/0} → gPVR (Fig. 4Bb) mice did not generate anti-OVA CTL responses when infected with polio-OVA. As expected, their CTLs were functional and generated a strong response to vaccinia-SS-SIINFEKL (data not shown). In contrast to the control B6 → gPVR mice, these TAP^{0/0} chimaeric mice also failed to generate CTL responses to a D^b -presented epitope from the polio VP0 protein (Fig. 4C). As shown above with vaccinia virus, these data indicate that non-haematopoietic cells are unable to stimulate

CTL immunity to another virus, indicating that this may be a general rule. These results also indicate that the bone-marrow-derived cells in B6 → c/gPVR mice acquired antigen from exogenous sources. That TAP^{0/0} → c/gPVR mice did not respond to either antigen also indicates that the response in B6 → c/gPVR mice was not due to residual PVR⁺ bone-marrow-derived cells that survived irradiation.

Until now, the physiological function of the exogenous MHC class I pathway has been unclear. Stimulation of CTLs by this route has been shown to occur in several situations, such as transplantation ('crosspriming' for minor histocompatibility antigens)¹⁶ and injection of particulate antigens¹⁷, but it has been thought to make a minor contribution to overall responses. Two situations in which this pathway has been shown to be important are in the generation of CTL responses to a tumour⁴ and in the homing and development of tolerance of adoptively transferred T cells specific for a transgenic antigen expressed in pancreatic β -cells^{18,19}. However, in these cases it was unclear whether the exogenous pathway might be dominant only because of the lack of inflammation (which stimulates antigen presentation and provides an adjuvant effect) and/or because these cells might be poor stimulators. It has been suggested that the exogenous MHC class I pathway is inefficient and unlikely to play an important part in most physiological situations²⁰. Our experiments with B6 → c/g PVR mice contradict this view and indicate that the exogenous MHC class I pathway is essential for the initiation of CTL

responses to viral infection that is confined to non-haematopoietic tissues. In fact, if this pathway did not exist, viruses could escape immune surveillance by using receptors that are not expressed on the critical, bone-marrow-derived APCs. Our results indicate that the presentation of exogenous antigen is a major pathway *in vivo* and may contribute to the stimulation of CTL responses even in situations in which viruses may infect bone-marrow-derived cells.

How do bone-marrow-derived cells acquire viral exogenous antigens? When infected cells die *in vivo*, they are rapidly cleared by bone-marrow-derived phagocytes, which will import the viral antigens into the exogenous MHC class I and class II pathways²¹. Interestingly, antigens from apoptotic cells are avidly presented on class I molecules of dendritic cells²². Although our results do not specifically establish the identity of the bone-marrow-derived APCs responsible for initiating CTL responses through the exogenous pathway, macrophages and/or dendritic cells are again the likely candidates, because they can present antigen through the exogenous MHC class I pathway *in vitro*^{17,23}. These cells can also ingest dying cells and cellular debris by phagocytosis and can thereby import viral antigens into the exogenous MHC class I pathway. Moreover, their migratory nature allows them to acquire antigen at a site of infection and then travel to the lymphoid tissues.

Two routes for the exogenous MHC class I pathway *in vitro* have been described, a TAP-independent pathway, in which antigen is probably hydrolysed in endosomes^{24,25}, and a phagosome-to-cytosol pathway²⁶ that is TAP-dependent. Our data provide indirect evidence that, *in vivo*, vaccinia-OVA and polio-OVA antigens may follow the TAP-dependent exogenous MHC class I pathway.

Our results show a strict requirement for professional APCs in the generation of anti-viral CTL immunity, and demonstrate that the exogenous pathway plays a key part in the immune surveillance of non-haematopoietic tissues. These findings have implications for vaccine delivery and gene therapy as well as for immune evasion by viruses. The results indicate that, to stimulate strong immunity, viral vectors or naked DNA must be expressed in professional APCs or delivered in a manner that will promote exogenous antigen presentation. Moreover, these mechanisms may limit the ability of viruses to block the generation of CTLs by downregulating MHC class I expression on infected cells²⁷, because this is unlikely to affect the exogenous pathway in uninfected professional APCs. □

Methods

TAP^{0/0} (B6, 129-Tap^{1p1Atp}; Jackson Laboratory) and B6 (Taconic) mice were obtained at 6–8 weeks of age. cPVR mice were made by standard transgenic techniques using the plasmid pVR-9, which has already been described¹⁴. gPVR mice (a gift from Cynamid) were bred at UMMC animal facilities. To prepare chimaeras, bone-marrow cells from 1–3-month-old donor mice were treated with anti-Thy1 antibody (M5/49.4.1; ATCC) and complement to eliminate mature T cells, washed twice and resuspended in PBS. Recipients were irradiated with 650 rad and then with 450 rad 4 h later. Irradiated mice were reconstituted by intravenous inoculation of $4\text{--}6 \times 10^6$ bone-marrow cells from the different donors. To avoid rejection of donor MHC class I-negative TAP^{0/0} cells by host natural-killer cells, chimaeras also received an intraperitoneal injection of 10 μ l rabbit anti-asialo GM1 gammaglobulin (Wako Chemicals) on the day of the transplant, and a second injection 3 days later. Bone-marrow chimaeras were rested for 4–6 months following reconstitution to allow for complete elimination of host-derived professional APCs. Mice were inoculated with virus and CTL killing was measured from fresh spleen cells (wild-type vaccinia), or from cultures restimulated with antigen for 5 days, using a ⁵¹Cr-release assay as described²⁸. For MHC class II-restricted responses, mice were injected at the base of the tail with 100 μ g OVA (Sigma) emulsified in complete Freund's adjuvant (Gibco) in a final volume of 50 μ l. Ten days later mice were killed and 2×10^5 cells from pooled para-aortic lymph nodes were incubated for 48 h in triplicate wells of microtitre plates in the presence of OVA. Supernatants were assayed for the presence of interleukin (IL)-2 by measuring the incorporation of ³H-thymidine by the IL-2-dependent cell line CTLL. When indicated, antibodies were added to culture as a 1:8 dilution of hybridoma culture supernatants. The antibodies M5/114, Y-3 and BBM.1 (ATCC) were used as anti-MHC class II, anti-MHC class I and control antibodies, respectively. All experiments were performed at least three times. Data points in all figures except Fig. 2 represent averages $\pm$ s.e.m. for three mice (all experimental groups) or two mice (vaccinia–SS-SIINFEKL; polio–sp27 controls). In Fig. 2, two mice were used per group and results from these mice are shown individually. All mice used in these experiments were housed at the UMMC animal facilities and experiments were conducted in compliance with NIH and institutional guidelines.

Viruses were produced and used as described^{14,28} except that inoculation of mice with polio was performed intravenously rather than intraperitoneally. For the generation of CTL responses to all viruses, the inoculation dose was 2×10^7 plaque-forming units (p.f.u.) per mouse diluted in 0.5 ml PBS. For the recovery of infectious virus from organs, mice were inoculated intraperitoneally with 2×10^8 p.f.u. of the poliovirus wild-type Mahoney 1 strain. Six paralysed cPVR mice were killed at days 4.5–6.5 after infection. Four control B6 mice were killed at days 4.5–5.5. Tissue samples were homogenized, and poliovirus titres in each tissue were determined by plaque assay.

P22 is a D^b-binding peptide corresponding to amino acids 22–30 of the poliovirus polyprotein that we have recently identified (L.J.S. and K.L.R., manuscript in preparation). Both SIINFEKL and P22 were synthesized at the peptide core facility at UMMC.

All cell lines used in this study have been described and were cultured as previously²⁸. To obtain dendritic cells, bone-marrow cells obtained from B6 mice were incubated overnight in RPMI media (Irvine Scientific) supplemented with 10% fetal calf serum (Atlanta Biologicals), 5×10^{-5} M 2-mercaptoethanol (Sigma) and 2 mM L-glutamine, antibiotics (Fungi-Bact), 0.01 M HEPES buffer and non-essential amino acids (all from Irvine Scientific). Non-adherent cells were collected and grown in the same media supplemented with 10 ng ml⁻¹ granulocyte/macrophage colony-stimulating factor and 5 ng ml⁻¹ IL-4 (Pharmingen) for 5–6 days with further addition of cytokines every other day. Most cells in these cultures were dendritic cells, as judged by morphology and analysis expression of specific markers by flow cytometry. Before being injected into mice, 3×10^6 dendritic cells were thoroughly washed in PBS, resuspended in 1 ml PBS containing the indicated concentrations of SIINFEKL and 10 μ g human β_2 -microglobulin (Calbiochem) and incubated at 37 °C for 1 h. Following incubation the cells were washed in PBS and injected intravenously into mice.

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Structure of the amino-terminal domain of Cbl complexed to its binding site on ZAP-70 kinase

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Cbl is an adaptor protein that functions as a negative regulator of many signalling pathways that start from receptors at the cell surface^{1–4}. The evolutionarily conserved amino-terminal region of Cbl (Cbl-N) binds to phosphorylated tyrosine residues and has cell-transforming activity. Point mutations in Cbl that disrupt its recognition of phosphotyrosine also interfere with its negative regulatory function and, in the case of v-cbl, with its oncogenic potential⁵. In T cells, Cbl-N binds to the tyrosine-phosphorylated inhibitory site of the protein tyrosine kinase ZAP-70⁶. Here we describe the crystal structure of Cbl-N, both alone and in complex

with a phosphopeptide that represents its binding site in ZAP-70. The structures show that Cbl-N is composed of three interacting domains: a four-helix bundle (4H), an EF-hand⁷ calcium-binding domain, and a divergent SH2 domain⁸ that was not recognizable from the amino-acid sequence of the protein. The calcium-bound EF hand wedges between the 4H and SH2 domains and roughly determines their relative orientation. In the ligand-occupied structure, the 4H domain packs against the SH2 domain and completes its phosphotyrosine-recognition pocket. Disruption of this binding to ZAP-70 as a result of structure-based mutations in the 4H, EF-hand and SH2 domains confirms that the three domains together form an integrated phosphoprotein-recognition module.

Cbl becomes tyrosine-phosphorylated upon engagement of several cell-surface receptors, including the multichain immune receptors, and growth-factor and cytokine receptors^{1–4}. The amino-acid sequence of Cbl reveals a RING-finger⁹ domain adjacent to the phosphotyrosine-binding Cbl-N segment, and a carboxy-terminal region with numerous docking sites for SH3- and SH2-containing proteins (Fig. 1). The oncogenic v-Cbl includes only the first 357 residues of Cbl and is a potent transforming protein¹⁰. The highly conserved Cbl-N region binds to the receptor for epidermal growth factor (EGFR)¹¹, Syk¹² and the negative-regulatory phosphorylation site in ZAP-70⁶. Genetic studies in *Caenorhabditis elegans* revealed that the Cbl homologue Sli-1 inhibits vulval induction by the EGFR¹³. Expression of D-Cbl, a *Drosophila melanogaster* Cbl homologue, disrupts EGFR-regulated development of the R7 photoreceptor¹⁴. Cbl also diminishes FcεRI-mediated degranulation in mast cells by inhibiting the tyrosine kinase Syk¹⁵. Mutational analysis demonstrates that Cbl-N is central to these functions. To understand better the diverse recognition and regulatory functions of Cbl-N, we have determined its three-dimensional structure.

As shown in Fig. 1, Cbl-N comprises three interacting domains: an N-terminal four-helix bundle (4H), a calcium-binding domain with the EF-hand fold, and an unusual SH2 domain. None of these folding motifs were previously recognized in the amino-acid sequence of Cbl. In spite of the structural and functional similarity of this unusual SH2 domain with other SH2 domains, it shares very little sequence identity (~11%) with them.

The N-terminal 4H domain contains four long α-helices. Structural comparisons with the DALI¹⁶ server show that it has a topology and overall structure similar to many functionally unrelated four-helical proteins, including cytochrome c, interleukin-5 and apolipoprotein III. The C and D helices in this domain pack against the adjacent EF-hand domain, and a highly conserved loop connecting the A and B helices contacts the SH2 domain.

Table 1 Data collection, phasing and refinement statistics

	Cbl-N	Cbl-N MeHg derivative	Cbl-N / ZAP70-292 complex
Resolution (Å)	15–2.20	15–2.24	20–2.10
Space group	C2	C2	P6
Unit cell (Å)	a=159.96 b=105.48 c=84.92 β=92.06°	a=160.04 b=106.73 c=84.84 β=92.30°	a= 122.3 c=55.65
Molecules/a.s.u.	3	3	1
R _{sym} (%)	6.7	5.5	7.9
Reflections (total/unique)	178,845/70,918	186,473/67,069	35,952/20,620
Completeness (%)	99.3	98.1	76.9 (91% to 2.5Å)
R _{iso} /R _{anom} (%)		35.1 / 6.5	
Phasing power (centric/accentric)		1.11 / 1.51	
R _{calc} (centric/accentric/anomalous %)		0.68 / 0.74 / 0.90	
FOM		0.41	
Number of sites		15	
Refinement statistics			
Resolution range (Å)	15–2.20		20–2.10
Non-hydrogen atoms	8,092		2,919
Water molecules	669		358
R _{cryst} / R _{free} (%)	22.1 / 28.7		17.3 / 24.6
R.m.s.d. bond lengths / angles	0.018Å / 2.1°		0.013Å / 1.84°

asymmetric unit (molecule B). Density for the other two molecules was broken and hard to interpret. The skeleton for molecule B was used to generate a molecular envelope for NCS averaging. Initial rotation matrices describing the relative orientations of molecules A, B and C were calculated from the heavy-atom sites (mercury bound to five sites in each of the three molecules). NCS averaging with DM²⁵ was used to improve phases at 2.7 Å resolution, and then extend phases to 2.2 Å, the limit of the native data set. The resulting electron density map was readily interpretable (Fig. 2). A model including residues 47–351 of each of the three molecules was built using the graphics program O²⁶. No electron density is seen for 24 residues at the amino terminus. The model was refined using simulated annealing and positional refinement in X-PLOR²⁷, with tight NCS restraints. The model includes 669 water molecules, which were positioned by using the program ARP (V.Lamzin). An overall thermal B factor and tightly restrained individual B factors were refined, and a bulk-solvent model was incorporated. Crystallographic R values and stereochemical parameters are presented in Table 1.

The structure of the Cbl-N/ZAP70 phosphopeptide complex was determined by molecular replacement with the program AmoRe²⁸. Use of the complete unliganded Cbl-N structure as a search model yielded clear rotation and translation peaks, and maps phased with the appropriately positioned model revealed strong electron density for the 4H and EF-hand domains, but no interpretable density for the SH2 domain. The model was therefore broken into two fragments (residues 47–265 and residues 266–351), and the rotation and translation searches were repeated with each fragment. The 4H/EF-hand fragment yielded a solution essentially identical to that from the intact model. The SH2 domain was then positioned using translation searches conducted in the context of the appropriately positioned 4H/EF-hand fragment. After rigid-body and positional refinement using X-PLOR²⁷, electron density maps calculated with the combined model revealed clear density for all domains, and readily interpretable density for the bound ZAP-70 phosphopeptide. After construction of the peptide, the structure was refined with iterative cycles of manual refitting and simulated annealing and positional refinement in X-PLOR (Table 1). Restrained individual temperature factors were refined. The model includes residues 47–351 of c-Cbl, residues 289–297 of ZAP-70, and 358 water molecules.

Illustrations

Figure 1a was prepared with MOLSCRIPT²⁹, Fig. 2 with program O²⁶, and Figs 1e and 3 with GRASP³⁰.

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Two subsets of memory T lymphocytes with distinct homing potentials and effector functions

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Naive T lymphocytes travel to T-cell areas of secondary lymphoid organs in search of antigen presented by dendritic cells^{1,2}. Once activated, they proliferate vigorously, generating effector cells that can migrate to B-cell areas or to inflamed tissues^{3–6}. A fraction of primed T lymphocytes persists as circulating memory cells that can confer protection and give, upon secondary challenge, a qualitatively different and quantitatively enhanced response^{7–9}. The nature of the cells that mediate the different facets of immunological memory remains unresolved. Here we show that expression of CCR7, a chemokine receptor that controls homing to secondary lymphoid organs, divides human memory T cells into two functionally distinct subsets. CCR7⁺ memory cells express receptors for migration to inflamed tissues and display immediate effector function. In contrast, CCR7⁺ memory cells express lymph-node homing receptors and lack immediate effector function, but efficiently stimulate dendritic cells and differentiate into CCR7⁺ effector cells upon secondary stimulation. The CCR7⁺ and CCR7⁺ T cells, which we have named central memory (T_{CM}) and effector memory (T_{EM}), differentiate in a step-wise fashion from naive T cells, persist for years after immunization and allow a division of labour in the memory response.

When blood-borne naive T cells home to lymph nodes, they first roll on high endothelial venules using CD62L. This allows the chemokine receptor CCR7 to engage its ligand SLC, which is displayed by endothelial cells¹⁰. The CCR7–SLC interaction activates integrins that promote firm adhesion and transmigration of

the T cells into the lymph node^{11,12}. In contrast to naive T cells, memory/effector cells migrate mostly through peripheral tissues¹³. This migration mediates rapid protective responses and is controlled by the expression of different sets of integrins and chemokine receptors^{1,14}. However, some memory T cells must also reach the lymph nodes to mount secondary proliferative responses. We considered whether the two facets of the memory response might depend on subsets of memory T cells invested with distinct homing and effector capacities.

Because CCR7 and CD62L are essential for lymphocyte migration to lymph nodes^{1,15}, the co-expression of these receptors might distinguish a putative subset of memory T cells that home to lymph nodes. Human naive and memory T cells can be identified by the reciprocal expression of the CD45RA or CD45RO isoforms¹⁶. Staining of peripheral blood T cells with antibodies to CD45RA and CCR7 revealed three subsets of CD4⁺ cells: one naive CD45RA⁺CCR7⁺; and two memory subsets, CD45RA⁺CCR7⁺ and CD45RA⁺CCR7⁺ (Fig. 1a). Both naive and CCR7⁺ memory cells expressed high levels of CD62L, whereas the CCR7⁺ memory cells expressed CD62L to a lower and variable extent (Fig. 1b). Within CD8⁺ T cells, the same three subsets could be identified, with an extra subset of CD45RA⁺CCR7⁺ cells (Fig. 1c). In addition, the two CCR7⁺ subsets expressed high levels of CD62L, whereas most of the cells among the two CCR7⁺ subsets lacked CD62L (Fig. 1d). This shows that lymph-node-homing receptors are expressed on a distinct subset of memory CD4 and CD8 T cells; furthermore, it identifies a subset of CD8⁺ cells that is CD45RA⁺, but that lacks both CCR7 and CD62L.

A number of chemokine receptors and adhesion molecules are involved in lymphocyte migration to secondary lymphoid organs or to tissues under homeostatic or inflammatory conditions^{1,14}. CCR7⁺ memory T cells express high levels of $\beta 1$ and $\beta 2$ integrins, which are required for homing to inflamed tissues¹⁷, as well as tissue-specific homing receptors such as CD103 and CLA (Table 1). Receptors for inflammatory chemokines, such as CCR1, CCR3 and CCR5, which have been found on memory/effector cells^{18,19}, were selectively

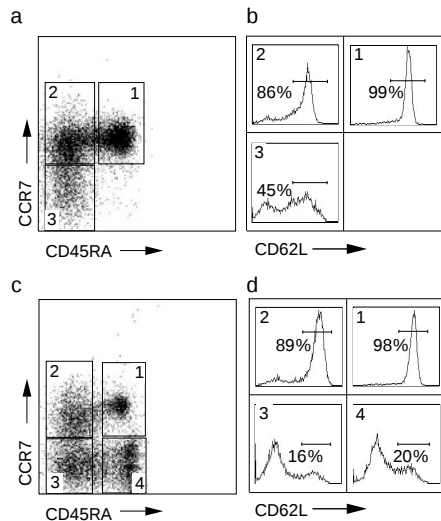


Figure 1 CCR7 and CD62L are co-expressed on a subset of peripheral blood memory CD4⁺ and CD8⁺ T cells. CD4⁺ (a, b) and CD8⁺ (c, d) lymphocytes were stained with monoclonal antibodies to CD45RA and CCR7, which identified three and four subsets, respectively. These subsets were sorted and analysed for the expression of CD62L, and the percentage of bright cells is indicated (b, d). Upon serial analysis, the proportion of cells in the different compartments was rather stable in the same individual, but more variable among individuals, the variability being more pronounced in the CD8 than in the CD4 compartment. Comparable results were obtained using two anti-CCR7 antibodies (clones 3D12 and 10H5).

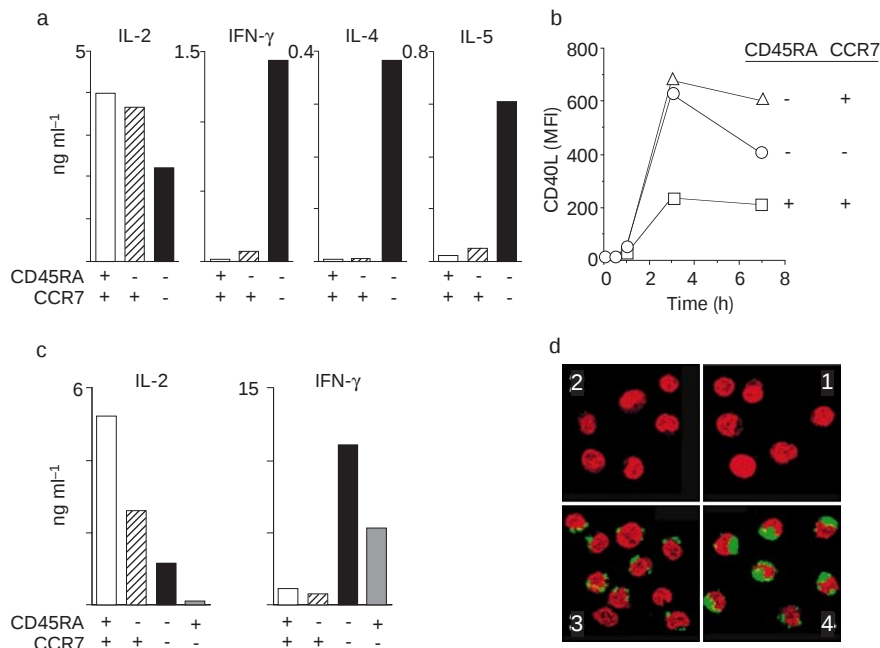


Figure 2 CCR7⁺ and CCR7⁺ memory T cells display different effector functions. a, b, The three subsets of CD4⁺ T cells were sorted according to the expression of CCR7 and CD45RA as in Fig. 1 and tested for their capacity to produce IL-2, IFN- γ , IL-4 and IL-5 (a) and for the kinetics of surface CD40L upregulation (b) following polyclonal stimulation. c, d, The four subsets of CD8⁺ T cells were sorted according to the expression of CCR7

and CD45RA as in Fig. 1 and tested for their capacity to produce IL-2 or IFN- γ (c) or were immediately stained with anti-perforin antibody (green) and counterstained with propidium iodide (red) (d). In the CD8⁺ CD45RA⁺ compartment, CCR7 expression allows us to discriminate naive cells (1) from effector cells (4) (ref. 26). Comparable results were obtained in 12 healthy donors.

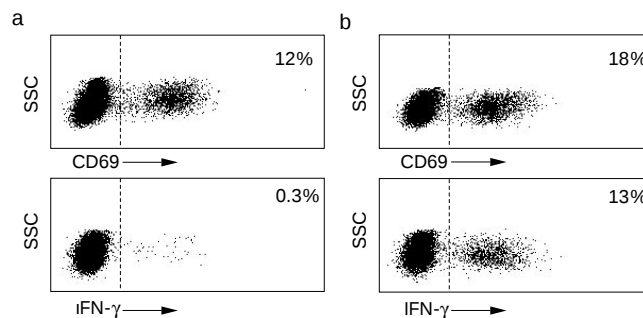


Figure 3 Rapid production of IFN- γ following stimulation of CCR7⁺ memory T cells. CD45RA⁺CCR7⁺ (a) and CD45RA⁺CCR7⁻ (b) CD4 T cells were stimulated for 7 h with

autologous dendritic cells pulsed with 100 ng ml⁻¹ TSST and stained with antibodies to CD69 and IFN- γ . CD69⁺ cells were less than 2% in unstimulated cultures.

expressed in the CCR7⁺ memory subset. On the other hand, CCR7⁺ memory cells had a distinct phenotype. They expressed intermediate levels of β 1 and β 2 integrins, as well as CCR4, CCR6 and CXCR3 on various proportions of cells. T-cell activation markers such as CD69 and CD25 were expressed only on a small fraction of the two memory subsets, whereas HLA-DR was expressed on about 10% of the CCR7⁺ memory cells. Together, these results imply that CCR7⁺ memory T cells share migratory routes with naive T cells, although the expression of additional receptors such as CCR4 may allow them to respond to a wider spectrum of chemokines and interact more effectively with dendritic cells^{20,21}.

Memory T cells carrying distinct homing receptors might participate in different types of immune responses and therefore might have different effector capacities. T-cell help for dendritic cells and B cells is dependent on expression of CD40L²², whereas protective responses in the tissues are mediated by T cells that produce effector cytokines, such as interferon- γ (IFN- γ) or interleukin-4 (IL-4), or release stored perforin^{23,24}. The naive and two memory CD4 subsets were sorted and compared for their capacity to produce cytokines and upregulate CD40L following stimulation. As shown in Fig. 2a, both naive T cells and CCR7⁺ memory cells produced IL-2 only. In contrast, the CCR7⁺ memory subset produced high levels of IL-4, IL-5 and IFN- γ and moderately reduced levels of IL-2. Upon activation, the extent of CD40L upregulation was comparable in

the two memory subsets and was higher than in naive T cells; however, the kinetics of upregulation were comparable, indicating that, unlike tonsil T cells²⁵, circulating memory T cells do not contain stored CD40L (Fig. 2b). Rapid production of IFN- γ was detected in most CCR7⁺, but only a negligible fraction of CCR7⁺ memory cells following stimulation with autologous dendritic cells pulsed with a bacterial superantigen (Fig. 3). Within the four CD8⁺ T-cell subsets, IFN- γ production and perforin-containing granules were restricted to the CCR7⁺ cells (Fig. 2c, d). Perforin expression was particularly prominent in the CD45RA⁺CCR7⁺ population that corresponds to a reported population of terminally differentiated CD27⁺ effector T cells²⁶. CD8⁺ cells progressively lost IL-2 production, presumably as a function of their differentiation from naive cells to effectors.

Table 1 Surface molecules on peripheral blood naive and memory CD4⁺ T-cell subsets

		CD45RA ⁺ CCR7 ⁺	CD45RA ⁺ CCR7 ⁺	CD45RA ⁺ CCR7 ⁻
CD3	(%)	>99	>99	>99
CD69	(%)	<0.1	2	3
CD25	(%)	<0.1	4	8
HLA-DR	(%)	<0.1	1	12
CD18	(MFI)	38	49	75
CD11a	(MFI)	90	140	218
CD11b	(%)	<0.1	<0.1	35
CD29	(MFI)	0	10/36*	43
CD49d	(MFI)	10	19/2	33/2
CD49e	(%)	<0.1	<0.1	10
CLA	(%)	<0.1	4	25
CD103	(%)	<0.1	<0.1	1
CXCR4	(%)	98	22	11
CCR4	(%)	<0.1	18	6
CCR6	(%)	<0.1	40	45
CXCR3	(%)	<0.1	34	61
CCR1	(%)	<0.1	1	14
CCR3	(%)	<0.1	<0.1	4
CCR5	(%)	<0.1	2	52

Three subsets of peripheral blood CDE4⁺ cells were sorted by expression of CD45RA or CD45RO and CCR7. The sorted cells were stained and analysed for the expression of adhesion molecules and chemokine receptors. MFI, mean fluorescence intensity.

* Mean value of major and minor peak.

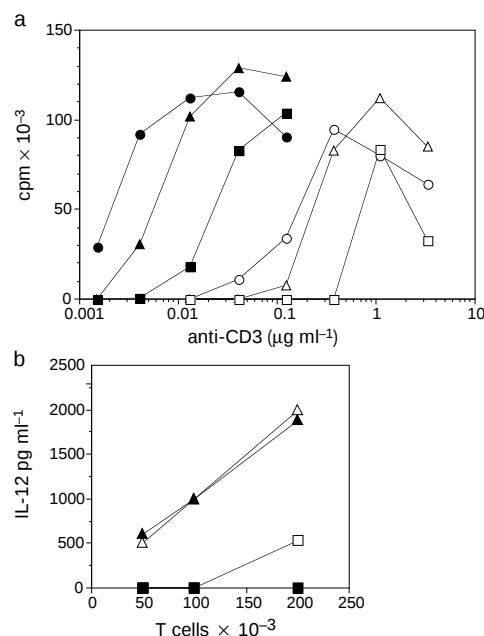


Figure 4 CCR7⁺ memory cells show enhanced responsiveness to T-cell receptor triggering and potently activate dendritic cells to produce IL-12. **a**, Proliferative response of naive T cells (squares), CCR7⁺ (triangles) and CCR7⁻ (circles) memory T cells to different concentrations of plastic-bound anti-CD3 monoclonal antibody in the absence (empty symbols) or in the presence (filled symbols) of anti-CD28. **b**, IL-12 p70 production by dendritic cells cultured with naive T cells (squares) or CCR7⁺ memory T cells (triangles). Dendritic cells were pulsed with toxic shock syndrome toxin (TSST) at 100 ng ml⁻¹ (empty symbols) or 1 ng ml⁻¹ (filled symbols). Both T-cell populations contained similar proportions of V β 2⁺ cells.

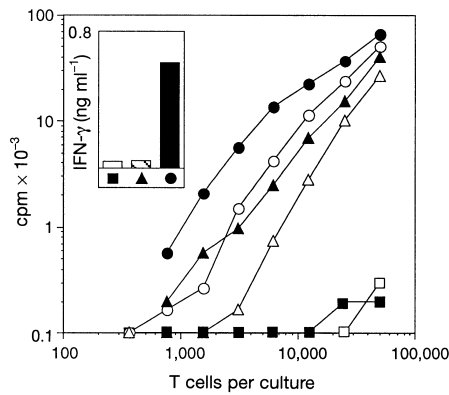


Figure 5 Proliferative responses to recall antigens can be detected in both CCR7⁺ and CCR7⁻ subsets but not in naive T cells. Proliferative response of CD45RA⁺ CCR7⁺ naive T cells (squares), CD45RA⁻ CCR7⁺ (triangles) and CD45RA⁻ CCR7⁻ memory T cells (circles) in response to tetanus toxoid presented by autologous monocytes. Responder cells from the same individual were tested 10 years after vaccination (empty symbols) and two weeks after a booster (filled symbols). Inset shows IFN- γ production in the 24-h culture supernatant using an input of memory cells giving comparable proliferative responses.

We then examined the activation requirements, which are less stringent for memory cells⁹. Compared with naive cells, both memory subsets displayed increased sensitivity to stimulation by anti-CD3, both in the presence and in the absence of costimulation, although CCR7⁻ cells were consistently more responsive (Fig. 4a). The capacity to stimulate IL-12 production was tested by co-culturing T cells with dendritic cells pulsed with different doses of the bacterial superantigen TSST. CCR7⁺ memory cells efficiently stimulated IL-12 production by dendritic cells at both high and low doses of TSST, whereas naive T cells were much less effective and only stimulated IL-12 at the highest TSST dose (Fig. 4b). Thus, because of their lower triggering threshold and higher capacity to upregulate CD40L, CCR7⁺ memory cells can function as potent activators of dendritic cells.

The above results indicated that two subsets of circulating memory T cells with different functional capacities can be discriminated by the expression of CCR7; therefore, we considered whether memory for antigens would be present in both subsets. As reported¹⁶, proliferative responses to tetanus toxoid can not be detected in naive T cells; however, they were consistently found in both the CCR7⁺ and CCR7⁻ memory subsets, even ten years after vaccination (Fig. 5). Following a booster with tetanus toxoid, the proliferative responses increased in both subsets, indicating that their relative proportions are not affected by recent antigenic stimulation. In all cases, IFN- γ was produced by only the CCR7⁻ memory cells. Comparable results were obtained by analysing the response to tetanus toxoid or hepatitis B surface antigen in five primed donors, showing that both memory subsets contain clonally expanded antigen-specific T cells, but these differ in their effector capacity. The presence of expanded antigen-specific memory cells in both subsets at different times after antigenic stimulation indicates that homeostatic mechanisms may maintain cells in both compartments^{16,27,28}.

What is the relationship between naive cells and the cells in the two memory subsets? When peripheral blood naive T cells were polyclonally stimulated, all cells became CD45RO⁺ after 10 days (data not shown), but most of the cells retained CCR7 expression, whereas only a few acquired the capacity to produce IL-4 or IFN- γ (Fig. 6a–c). When the CCR7⁺ and CCR7⁻ cells were sorted and stimulated, IL-4, IL-5 and IFN- γ were found to be exclusively produced by the CCR7⁻ cells (Fig. 6d). Thus, with respect to the

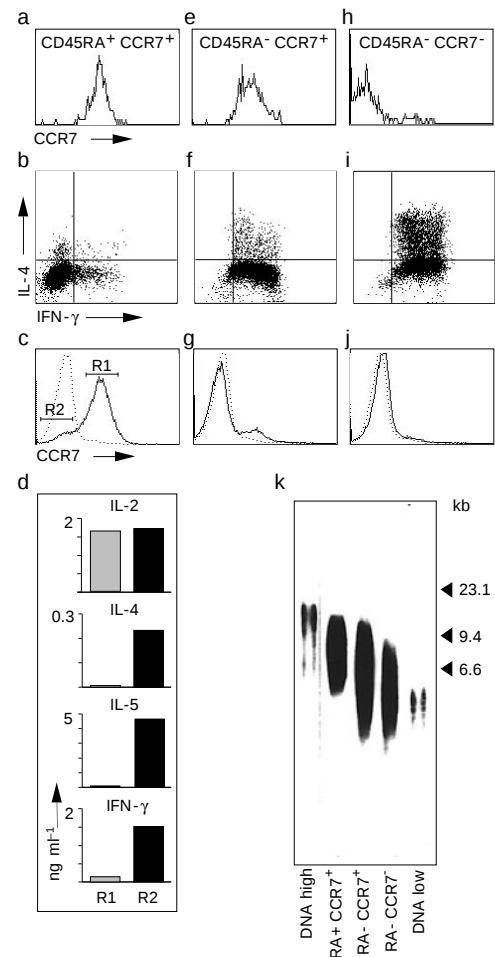


Figure 6 Differentiation potential of naive and memory T-cell subsets. **a–d**, Loss of CCR7 following *in vitro* stimulation of naive T cells correlates with acquisition of effector function. CD4⁺ naive T cells (CD45RA⁺, CCR7⁺) were sorted from peripheral blood (**a**), stimulated with anti-CD3 + anti-CD28, expanded for 10 days in IL-2 and tested for their capacity to produce IFN- γ and IL-4 (**b**) or for CCR7 expression (**c**). CCR7⁺ (R1) and CCR7⁻ cells (R2) were sorted and immediately tested for their capacity to produce cytokines following polyclonal stimulation (**d**). **e–g**, Rapid polarization of CCR7⁺ memory T cells following *in vitro* stimulation. CD4⁺, CD45RA⁻, CCR7⁺ T cells were sorted from peripheral blood (**e**), stimulated, expanded and tested for cytokine production (**f**) and CCR7 expression (**g**). **h–j**, CD4⁺, CD45RA⁻, CCR7⁻ T cells isolated and stimulated as above retained a stable effector phenotype. **k**, Length of telomeres in peripheral blood naive and memory CD4⁺ T-cell subsets. kb, kilobases.

parameters analysed, it appears that the same functional subsets of memory T cells that are detected *in vivo* can be generated by stimulation of naive cells in short-term culture.

When peripheral blood CCR7⁺ memory T cells were sorted and stimulated under the same conditions, almost all the cells that were recovered after 10 days had lost CCR7 expression and had acquired the capacity to produce effector cytokines upon further stimulation (Fig. 6e–g), indicating that this subset is poised to generate effector cells. Finally, peripheral blood CCR7⁻ memory cells, after stimulation and expansion, retained their CCR7⁻ phenotype and effector function (Fig. 6h–j). This indicates that, at least *in vitro*, there may be a stepwise differentiation from naive T cells to CCR7⁺ memory to CCR7⁻ memory/effector T cells. This possibility is supported by analysis of the telomere length, which decreases as a function of cell division²⁹. As shown in Fig. 6k, the length of telomeres in peripheral blood CD4 subsets decreased progressively from naive to memory

cells, but was consistently higher in the CCR7⁺ than in the CCR7⁻ subset, suggesting that the latter had undergone a larger number of divisions.

We have shown that immunological memory is displayed by distinct T-cell subsets: lymph-node-homing cells lacking inflammatory and cytotoxic function (which we define as central memory T cells, T_{CM}) and tissue-homing cells endowed with various effector functions (which we define as effector memory T cells, T_{EM}). These two subsets allow a division of labour among memory cells. On the one hand, T_{EM} cells represent a readily available pool of antigen-primed cells which can enter peripheral tissues to mediate inflammatory reactions or cytotoxicity, thus rapidly containing invasive pathogens. On the other hand, the newly described T_{CM} cells represent a clonally expanded antigen-primed population which travels to secondary lymphoid organs and, upon a secondary challenge, can efficiently stimulate dendritic cells, help B cells and generate a new wave of effector cells.

Our results indicate a precursor-product relationship between the two memory subsets. *In vitro* stimulation of naive T cells results in the generation of both T_{CM} and T_{EM} cells, whereas stimulation of T_{CM} cells results in their efficient differentiation to T_{EM} cells. Furthermore, antigen-specific T_{CM} and T_{EM} cells persist *in vivo* for up to ten years and their relative proportions do not change after a booster immunization. These data are consistent with a linear differentiation model in which naive T cells differentiate first to T_{CM} and then to T_{EM} cells, depending on the strength and duration of T-cell receptor stimulation and the presence or absence of polarizing cytokines²¹. Understanding the mechanisms that generate and maintain the two types of memory cells will help to manipulate immunological memory for vaccination and for adoptive immunotherapy. □

Methods

Sorting and FACS analysis

Peripheral blood mononuclear cells were stained with a rat monoclonal antibody (mAb) specific for CCR7 (3D12, IgG2a) followed by a fluorescein isothiocyanate (FITC)-labelled mouse anti-rat IgG2a mAb (PharMingen) or alternatively by a phycoerythrin (PE)-labelled goat anti-rat immunoglobulin polyclonal antiserum (Southern Biotechnology Associates). The 3D12 mAb completely inhibited migration of peripheral blood T cells in response to secondary lymphoid tissue chemokine (SLC) and EB11-ligand chemokine (ELC) (R.F., unpublished data) and did not affect the response of T cells to mitogenic or antigenic stimulation (F.S., unpublished data). In addition, 3D12 stained all cell lines that expressed CCR7 messenger RNA, but did not stain CCR7 mRNA-negative cells. In some experiments, a mouse mAb specific for CCR7 (10H5, IgG3; produced by L. Wu, LeukoSite) was used with comparable results. The following PE-, PC5- or APC-labelled mouse mAbs were used in different combinations: anti-CD45RA (ALB11, IgG1); anti-CD45R0 (UCHL1, IgG2a); anti-CD3 (UCHT1, IgG1); anti-CD4 (13B8.2, IgG1); anti-CD8 (B9.11, IgG1); anti-CD11a (25.3, IgG1); anti-CD11b (Bear1, IgG1); anti-CD18 (7E4, IgG1); anti-CD49d (HP2/1, IgG1); anti-CD49e (SAM1, IgG2b); anti-CD29 (K20, IgG2a); anti-CD103 (2G5, IgG2a); anti-CD69 (TP1.55, IgG2b); anti-CD25 (B1.49, IgG2a); anti HLA-DR (B8.12, IgG2b); anti-CD40L (TRAP-1, IgG1) (all from Immunotech); and anti-CLA (HECA-205, rat IgG1; PharMingen). Staining for chemokine receptors was carried out using the following mouse mAbs (all produced at LeukoSite): anti-CCR1 (2D4, IgG1); anti-CCR3 (7B11, IgG2a); anti-CCR4 (1G1, IgG1); anti-CCR5 (2D7, IgG2a); anti-CCR6 (11A9, IgG1); anti-CXCR3 (1C6, IgG1); and anti-CXCR4 (12G5, IgG2a). Cells were sorted using a fluorescence-activated cell sorter (FACS Vantage) and analysed on a FACScalibur (Becton Dickinson Systems). Sorted cells were immobilized on poly-L-lysine coated slides, fixed in 2% paraformaldehyde and permeabilized in 0.1% Triton-X100 before intracellular staining with an anti-perforin mAb (284, IgG2b; PharMingen), followed by FITC-labelled goat anti-mouse immunoglobulin and propidium iodide to visualize the nuclei by confocal microscopy. The length of telomeres was determined using a Teloquant kit (PharMingen).

Cytokine detection

T cells were stimulated with 10 µg ml⁻¹ anti-CD3 antibody (TR66, IgG1) and 10⁻⁷ M phorbol 12-myristate 13-acetate (PMA; Sigma). Cytokine production was measured in the 24-h culture supernatants by ELISA using matched pairs of antibodies specific for IL-2, IL-4, IL-5, IFN-γ (PharMingen). For cytokine detection at the single-cell level, T cells were stimulated with 10⁻⁷ M PMA and 1 µg ml⁻¹ ionomycin for 4 h, or with autologous dendritic cells pulsed with 100 ng ml⁻¹ TSST for 7 h in 10 µg ml⁻¹ brefeldin A. Cells were fixed and permeabilized with PBS containing FCS (2%) and saponin (0.5%) and stained with FITC-labelled anti-IFN-γ (IgG1) and PE-labelled anti-IL-4 (IgG2b) or PE-labelled anti-CD69 mAbs.

Cell cultures

Sorted cells were stimulated with plastic-bound anti-CD3 (TR66) and anti-CD28 (CD28.1, IgG1; provided by D. Olive) in RPMI 1640 medium containing 2 mM L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 50 µg ml⁻¹ kanamycin, (complete medium; Gibco BRL) and 10% FCS (HyClone Laboratories). The cells were expanded with 500 U ml⁻¹ IL-2. Monocytes were isolated using magnetic beads coated with anti-CD14 mAb (Miltenyi), irradiated (3,000 rad), pulsed with 5 µg ml⁻¹ TT and cultured with different numbers of T cells. IFN-γ was measured by ELISA in the 24-h culture supernatant. ³H-thymidine incorporation was measured on day five. Immature dendritic cells were produced by culturing peripheral blood CD14⁺ monocytes with GM-CSF + IL-4 for six days. The cells were pulsed with different concentrations of TSST and cultured with graded numbers of T cells. IL-12 p70 was measured by ELISA (PharMingen) in the 24-h culture supernatant.

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The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens

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Macrophages orchestrate innate immunity by phagocytosing pathogens and coordinating inflammatory responses¹. Effective defence requires the host to discriminate between different pathogens. The specificity of innate immune recognition in *Drosophila* is mediated by the Toll family of receptors^{2,3}; Toll mediates anti-fungal responses, whereas 18-wheeler mediates anti-bacterial defence^{4–6}. A large number of Toll homologues have been identified in mammals, and Toll-like receptor 4 is critical in responses to Gram-negative bacteria^{7–11}. Here we show that Toll-like receptor 2 is recruited specifically to macrophage phagosomes containing yeast, and that a point mutation in the receptor abrogates inflammatory responses to yeast and Gram-positive bacteria, but not to Gram-negative bacteria. Thus, during the phagocytosis of pathogens, two classes of innate immune receptors cooperate to mediate host defence: phagocytic receptors, such as the mannose receptor, signal particle internalization, and the Toll-like receptors sample the contents of the vacuole and trigger an inflammatory response appropriate to defence against the specific organism.

Macrophage phagocytosis of the yeast cell-wall particle zymosan is accompanied by secretion of the inflammatory mediator tumour necrosis factor- α (TNF- α). Internalization of zymosan is mediated, in part, by the mannose receptor, a classic example of an innate immune pattern-recognition molecule, but the signalling pathway that elicits TNF- α production is unknown¹². Given the role of Toll-like receptors in pro-inflammatory signalling during innate defence, we explored whether these molecules are also found on phagosomes. We expressed haemagglutinin A (HA)-tagged murine Toll-like receptor 2 (TLR2) in the mouse macrophage cell line, RAW-TT10. In the absence of a phagocytic stimulus, the receptor was distributed uniformly on the cell surface (Fig. 1a). After a 5-min incubation with zymosan particles, TLR2 was highly enriched on phagosomes (Fig. 1b, c). Phagocytosis occurs through a series of discrete steps: initially the particle is bound to the cell surface; actin polymerization leads to pseudopod extension around the particle; the particle is completely engulfed by the cell; and lastly actin is depolymerized, allowing further phagosome maturation through membrane-transport events¹. The enrichment of TLR2 was apparent throughout all phases of internalization (Fig. 1b, c, zymosan particles 1–4): the receptor was enriched under bound particles (zymosan 1), in processes extending around extracellular particles (zymosan 2) and in membranes around fully engulfed particles (zymosan 3), and it remained enriched on the phagosome for several minutes after actin disassembly (zymosan 4; and data not shown). Within 10 min after internalization, however, TLR2 was no longer enriched on the vacuole (data not shown).

Phagocytosis of particles is tightly coupled to the production of pro-inflammatory cytokines, but the mechanism by which this occurs is unknown. The presence of TLR2 on phagosomes indicated that it might mediate pro-inflammatory signals during zymosan phagocytosis. To establish a role for TLR2 in zymosan-induced cytokine release, we needed to define a dominant-negative form of

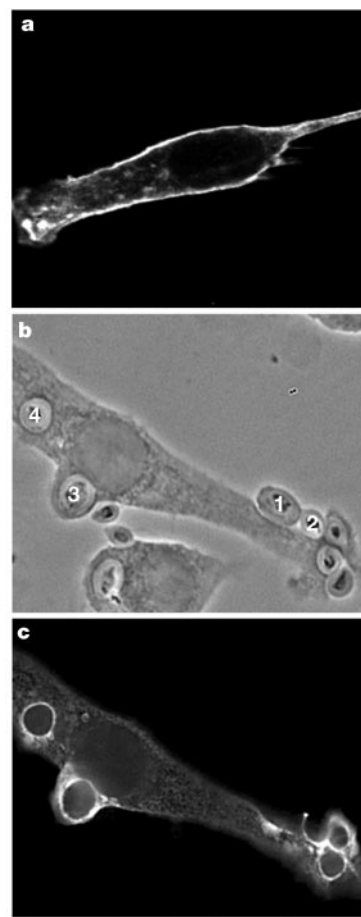


Figure 1 TLR2 is enriched on phagosomes containing yeast particles. **a**, Epitope-tagged TLR2 was detected on the cell surface by immunofluorescence microscopy in RAW-TT10 cells transiently expressing the receptor. **b**, When these cells were incubated with zymosan for 5 min and processed for immunofluorescence microscopy, zymosan particles, both external (particles 1 and 2) and internal (particles 3 and 4), were clearly visible by phase microscopy. **c**, TLR2 was markedly enriched around the particles during all stages of internalization.

this receptor. Genetic evidence in the C3H/HeJ mouse indicates that a P712H substitution in the related Toll-like receptor 4 (TLR4) may act as a dominant-negative mutation of the receptor and prevent LPS signalling in the mouse^{10,11,13}. We therefore constructed a putative dominant-negative mutant TLR2, in which proline 681 was replaced by a histidine (TLR2-P681H), a mutation that is equivalent to the P712H substitution in TLR4. Chinese hamsters fail to express functional TLR2 because of a frame-shift mutation in the receptor¹⁴, making Chinese hamster ovary (CHO) cells an ideal model system in which to define dominant-negative mutants of TLR2 signalling. Expression of TLR2 in CHO cells resulted in a moderate level of constitutive activation of NF- κ B, which was not increased substantially upon exposure of the cells to zymosan (Fig. 2a). CD14 has been shown to facilitate the activation of TLR2 by bacterial products^{15,16}. Cotransfection of CHO cells with CD14 and TLR2 greatly enhanced zymosan-induced NF- κ B activation (Fig. 2a), whereas expression of CD14 by itself did not mediate zymosan-induced signalling (data not shown). TLR2-P681H did not mediate zymosan-induced activation of NF- κ B in the presence of CD14, indicating that the mutation abrogated the capacity of the receptor to signal (Fig. 2a). Importantly, TLR2-P681H acted as a dominant-negative inhibitor of TLR2-mediated activation of NF- κ B (Fig. 2a). Identical results were obtained with *Staphylococcus aureus* (data not shown). When expressed in RAW-TT10 macro-

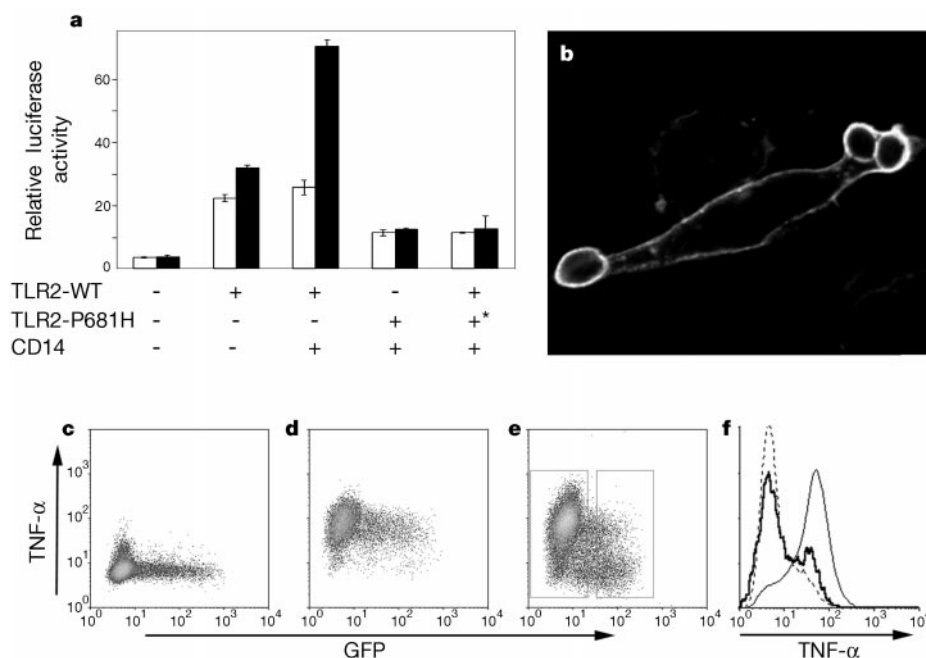


Figure 2 Expression of TLR2-P681H blocks zymosan-induced TNF- α production. **a**, The relative activity of a NF- κ B-luciferase reporter was assessed in CHO cells transiently expressing the indicated constructs before (open bars) or after (filled bars) stimulation with zymosan. In the sample showing inhibition of TLR2 by TLR2-P681H (asterisk), the mutant receptor was cotransfected with the wild-type receptor at a 10:1 ratio. **b**, Epitope-tagged TLR2-P681H, transiently expressed in RAW-TT10 cells, was highly enriched on zymosan phagosomes. The HA tag was detected by immunofluorescence microscopy as in Fig. 1. **c**, **d**, RAW-TT10 cells transiently transfected with the control expression vector (pTIGZ2+)

were stained for production of TNF- α and analysed by flow cytometry following no stimulation (**c**), or after incubation with zymosan (**d**). **e**, Cells expressing TLR2-P681H failed to induce TNF- α after stimulation with zymosan. **f**, Using the regions defined in **e**, the thin line indicates TNF- α produced in stimulated GFP-low cells, and the bold line indicates TNF- α produced in stimulated, TLR2-P681H-expressing cells (GFP-high). The dotted line indicates background, unstimulated TNF- α production; y axis, relative cell number.

phages, TLR2-P681H was distributed at the cell surface and was substantially enriched around zymosan-containing phagosomes, indicating that it was properly localized to function as a dominant-negative inhibitor of particle-induced signalling through endogenous TLR2 (Fig. 2b).

To determine whether TLR2-P681H was capable of inhibiting the production of pro-inflammatory cytokines during phagocytosis in macrophages, we designed a single-cell assay in which we could simultaneously analyse the expression of the mutant receptor and zymosan-induced TNF- α production. In this assay, transient expression of TLR2-P681H and green fluorescent protein (GFP) from a single bicistronic transcript permitted GFP intensity (as measured by flow cytometry) to reflect accurately the level of TLR2-P681H expression (see Supplementary Information). RAW-TT10 cells were transfected with a control vector expressing only GFP, and 100,000 cells were analysed by flow cytometry. TNF- α production remained at background levels over three logs of expression of GFP (Fig. 2c). When stimulated with zymosan particles, TNF- α production was induced equally in all cells irrespective of their level of GFP expression (Fig. 2d). Expression of TLR2-P681H correlated in a dose-dependent manner with the inhibition of TNF- α production induced by zymosan (Fig. 2e). Cells producing TLR2-P681H (as measured by GFP expression) clearly failed to produce TNF- α , whereas cells in the same sample that did not express the inhibitory receptor produced TNF- α . Expression of TLR2-P681H in RAW-TT10 cells also inhibited zymosan-induced activation of NF- κ B, as measured by its translocation from the cytosol to the nucleus (see Supplementary Information). Particle binding and internalization were unaffected by the expression of the mutant receptor (data not shown; and see below), indicating that phagocytosis and particle-induced cytokine production were uncoupled in these cells. The full spectrum of TNF- α production by GFP-positive or GFP-negative cells (Fig. 2e, boxed regions) is shown in Fig. 2f. GFP-negative cells

(Fig. 2f, thin line) had appreciable zymosan-induced TNF- α production, which was inhibited in TLR2-P681H-expressing cells (thick line), effectively to unstimulated levels (dotted line). Thus, TLR2 couples the phagocytosis of zymosan to TNF- α production, and the extent of the inhibition (>90%) suggests that this is the primary pathway.

As phagocytosis of yeast resulted in TLR2-mediated signalling, we examined the potential for this receptor to signal TNF- α production during the phagocytosis of other pathogens. TNF- α production induced during the phagocytosis of *S. aureus*, a Gram-positive bacterium, was completely inhibited by the expression of TLR2-P681H (Fig. 3), as was TNF- α induction by the Gram-positive bacterial cell-wall components lipoteichoic acid and peptidoglycan (data not shown). By contrast, TNF- α production elicited during the phagocytosis of *Salmonella minnesota* R595, a Gram-negative bacterium, was unaffected by the expression of TLR2-P681H (Fig. 3). Similarly, lipopolysaccharide (LPS), a key pro-inflammatory component of the Gram-negative bacterial cell wall, induced TNF- α normally in the presence of TLR2-P681H (Fig. 3). Thus, TLR2 discriminates yeast and Gram-positive bacteria from Gram-negative bacteria. The C3H/HeJ mouse, which bears a P712H mutation in TLR4, is hyporesponsive to LPS, indicating that TLR4 participates in the recognition of Gram-negative bacteria^{10,11}. Expression of TLR4-P712H in RAW-TT10 macrophages strongly inhibited LPS-induced TNF- α production, whereas it had no effect on *S. aureus*-induced signalling (Fig. 3). TLR4-P712H inhibited *S. minnesota*-induced TNF- α production to a lesser degree than was observed with soluble LPS (Fig. 3). These data show that individual Toll family receptors convey specificity in mediating pro-inflammatory signals from different pathogens.

Further insight into the mechanism by which TLR2 signals was obtained by the observation that expression of the wild-type receptor partially inhibited zymosan- and *S. aureus*-induced

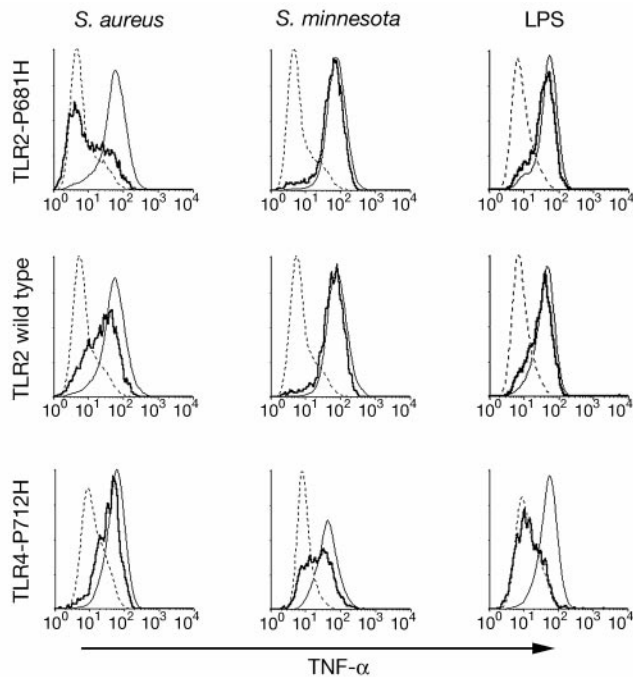


Figure 3 Inhibition of particle-induced TNF- α production by TLR2-P681H is selective. RAW-TT10 cells transiently transfected with TLR2-P681H (upper panels), wild-type TLR2 (middle panels) or TLR4-P712H (lower panels) were analysed for particle-induced TNF- α production. The cells were stimulated as indicated with *S. aureus*, *S. minnesota* or LPS. Using the regions defined in Fig. 2e, the thin lines indicate TNF- α produced in stimulated, GFP-low cells, and the bold lines indicate TNF- α produced in stimulated, TLR-expressing cells (GFP-high). The dotted lines indicate background, unstimulated TNF- α production; y axes, relative cell number.

TNF- α production, but had no effect on *S. minnesota*- and LPS-induced TNF- α production (Fig. 3; and data not shown). The data indicate that over-expression of the wild-type receptor may dilute out other molecules required for TLR2 function, perhaps a structural component of the receptor, and further shows that TLR2 does not participate in the response of macrophages to Gram-negative bacteria.

MyD88 is a cytoplasmic adaptor protein that binds to the Toll-homology domain of TLR4 and the interleukin-1 receptor (IL-1R), and mediates their downstream signalling^{17–20}. Binding of MyD88 to these receptors is mediated by a C-terminal Toll-homology domain, whereas its N-terminal death domain is important for the propagation of the signal through the protein kinase IRAK (IL-1 receptor associated kinase). The C-terminal Toll domain of MyD88 is a dominant-negative inhibitor of TLR4 and IL-1R signalling, whereas the death domain constitutively activates these pathways^{17–19}. Expression of the C-terminal MyD88 dominant negative (MyD88-DN) correlated precisely with inhibition of TNF- α production induced during the phagocytosis of zymosan and the Gram-positive bacterium, *S. aureus* (Fig. 4a). The failure of TNF- α induction could not be attributed to an inhibition of particle binding or internalization, as the internalization of fluorescently labelled zymosan, *S. aureus* and *S. minnesota* was unaffected by MyD88-DN expression (Fig. 4b). Thus, MyD88 is required for TLR2 signalling in the macrophage. MyD88-DN also inhibited TNF- α production induced by the Gram-negative bacterium *S. minnesota* and by LPS (Fig. 4a), although the inhibitor was less effective against the whole bacterium (as observed with TLR4-P712H in Fig. 3). These later data indicate that, during phagocytosis of Gram-negative bacteria, a MyD88-dependent pathway (presumably

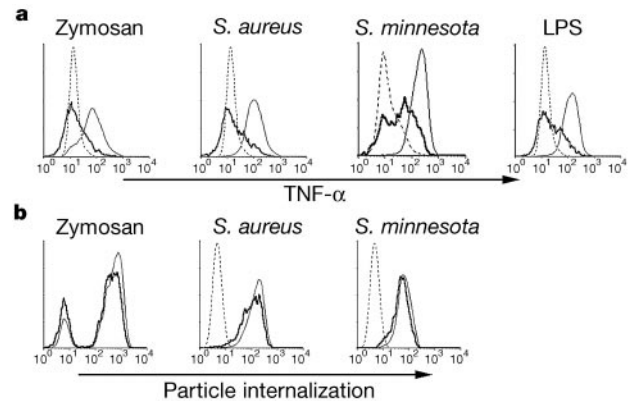


Figure 4 Expression of MyD88-DN blocks particle-induced TNF- α production but not particle internalization. **a**, RAW-TT10 cells transiently expressing MyD88-DN were stimulated as indicated with zymosan, *S. aureus*, *S. minnesota* or LPS, and TNF- α production was measured. Using the regions as defined in Fig. 2e, the thin lines indicate TNF- α produced in stimulated, GFP-low cells, and the bold lines indicate TNF- α produced in stimulated, MyD88-DN-expressing cells (GFP-high). The dotted lines indicate background, unstimulated TNF- α production. **b**, Cells transiently expressing MyD88-DN were incubated with fluorescently labelled zymosan, *S. aureus*, or *S. minnesota* as indicated, and particle internalization was assessed by flow cytometry; y axes, relative cell number.

initiated by TLR4) and a MyD88-independent pathway may contribute to the complete induction of TNF- α . This hypothesis concurs with the observation that, although the TLR4 mutation in the C3H/HeJ mouse blocks soluble LPS signalling, Gram-negative bacteria still stimulate cytokine production (perhaps through Toll-independent pathways)²¹. Thus, both genetic data and our results indicate that TLR4 may be the primary mediator of LPS signalling, whereas our data show that TLR2 principally mediates signals from yeast and Gram-positive bacteria in macrophages. Previously, human TLR2 has been implicated in LPS signalling in non-macrophage cell lines, and it not clear why our data, obtained in cells that are naturally LPS-responsive, differ^{22,23}; however, our data concur with genetic evidence in mice and hamsters, in which a mutation in TLR4 abrogates LPS signalling whereas a mutation in TLR2 does not^{10,11,14}. We have also shown that the natural mutation in the TLR4 gene in the C3H/HeJ LPS-hyporesponsive mouse (P712H) acts as a dominant-negative inhibitor of TLR4 function. Notably, the corresponding proline-to-histidine mutation in TLR2 (P681H) also acts as a dominant-negative mutation in TLR2. This proline is conserved in all known mammalian Toll receptors, except TLR3 (ref. 7–9), and might couple Toll receptors to a common signalling pathway.

Macrophages are capable of detecting the nature of the pathogen within the phagocytic vacuole, but the mechanisms by which this occurs are unknown¹. Our data indicate that the toll-like receptors may be recruited to phagosomes, where they sample the contents and determine the nature of the pathogen; TLR2 detects Gram-positive bacteria and fungi, whereas TLR4 detects Gram-negative bacteria. Thus, specific Toll-like receptors may distinguish the contents of the phagosome and participate in the elaboration of an inflammatory response appropriate for defence against a specific pathogen. □

Methods

Murine TLR2 cloning

Two mouse ESTs (Genbank AA863729 and D77677) with homology to the human TLR2 sequence were identified, and a 0.9-kilobase (kb) complementary DNA was generated by reverse transcription of RAW 264.7 total RNA using a primer spanning the stop codon in

the first EST (5'-GGTGGAGAACCTAGGACTTTATTGC-3'). Polymerase chain reaction (PCR) was carried out with the same primer, and the 5' most homologous sequences in the second EST (5'-GCTCTATATTCAGAAATAAGCTG-3'). To obtain the complete open reading frame (ORF), 5' rapid amplification of complementary DNA ends (RACE) was performed on RAW 264.7 RNA (Gibco-BRL). Products from 0.5 to 1.5 kb were amplified, cloned and sequenced, completing the murine TLR2 ORF (Genbank AF185284).

DNA expression vectors

The mammalian expression vector, pNeo/Tak, was constructed to combine expression of the tetracycline transactivator²⁴ from a tetracycline-regulated promoter (pTet-Tak, Gibco-BRL) with neomycin selection. The neomycin resistance marker was from pcDNA3 (Invitrogen), and the remainder of the plasmid backbone was derived from pBluescript SK (Stratagene).

The expression vector pTIGZ2+ was assembled to direct tetracycline-regulated expression of a protein from the same messenger RNA transcript as GFP. In this vector, the tetracycline-regulated promoter from pTetSplice (Gibco-BRL) directed expression of a mRNA encoding the protein of interest followed by the cap-independent translational enhancer region of pCITE (Novagen) driving translation of enhanced GFP (from pEGFP-N1, Clontech). The polyadenylation signal and remainder of the vector backbone were from pcDNA3.1/Zeo (Invitrogen). During construction, the tetracycline-inducible promoter was modified to contain two binding sites for the tetracycline transactivator instead of seven.

The HA-TLR2 expression vector was constructed by amplifying the coding region of mouse TLR2 from amino acids R21–S784 (removing the endogenous signal peptide) by PCR, and cloning the fragment into a modified pDisplay (Invitrogen) which provides a signal peptide and a haemagglutinin A epitope. pDisplay had been modified by deletion of the PDGF transmembrane domain and replacement of the neomycin resistance cassette by one with zeocin resistance (pcDNA3.1/Zeo, Invitrogen). For single-cell TNF- α measurements, the HA-tagged TLR2 coding region was transferred from pDisplay to TIGZ2+. The TLR2 dominant-negative P681H mutation was generated by PCR and confirmed by sequencing. The MyD88 dominant-negative construct was generated by amplifying the MyD88 C-terminal coding region (amino acids 146–296) by PCR from cDNA of RAW 264.7 cells and cloning the fragment into TIGZ2+. The murine TLR4 coding region was amplified by RT-PCR from RAW cells (Genbank AF185285), and cloned into pTIGZ2+. The dominant-negative P712H mutation was generated by PCR and confirmed by sequencing, and the resulting coding region was cloned into pTIGZ2+. Murine CD14 was amplified by RT-PCR from RAW cells and cloned into the expression vector pcDNA3.1/Zeo (Invitrogen).

Transfection

RAW 264.7 (ATCC# TIB-71) and CHO-K1 (ATCC# CRL-9618) cells were transfected as described²⁵. All experiments were performed 24 h after transfection. For generation of a tetracycline transactivator-expressing RAW cell line, RAW 264.7 cells were transfected with pNeo/Tak, and stable cell lines were selected using 400 $\mu\text{g ml}^{-1}$ G418 (Gibco-BRL). After 10 days of selection, the cells were cloned by limiting dilution, and one cell line (designated RAW-TT10) that showed good tetracycline-regulated expression from a subsequently transfected reporter plasmid was used for all experiments. Tetracycline was always absent from the media, resulting in strong activity of the tetracycline-regulated promoter.

Immunofluorescence microscopy

RAW cells were processed for immunofluorescence microscopy as described²⁵. Cells grown on glass coverslips were incubated for 5 min at 37 °C with zymosan (Molecular Probes), fixed and permeabilized, and nonspecific sites were blocked by incubation in blocking buffer (PBS, 10% calf serum, 0.1% ovalbumin and 5 mM sodium azide). HA-tagged TLR2 was detected using a mouse monoclonal antibody (HA.11, Babco) precleared against zymosan. Specific binding was detected with fluorescein-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch). After mounting, the cells were observed by fluorescence microscopy using an Axiovert TV microscope (Carl Zeiss) equipped with a cooled CCD camera (Princeton Instruments) and Metamorph imaging software (Universal Imaging).

Luciferase assays

CHO-K1 cells were transiently transfected with 2 μg of NF- κB reporter construct (ELAM-1 firefly luciferase²⁶) and 0.2 μg of a construct directing expression of *Renilla* luciferase under control of the constitutively active β -actin promoter²⁷, together with 3 μg of each of the expression constructs indicated in the text, and plated into 24-well tissue-culture plates. Twenty-four hours after transfection, the cells were stimulated with 3×10^6 zymosan particles (Molecular Probes) per well for 5 h, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Data are presented as the mean $\pm$ standard deviation of triplicate samples and are representative of three independent experiments.

Detection of intracellular TNF- α

S. aureus (clinical isolate) and *S. minnesota* (ATCC#49284) were grown to saturation, heat killed and stored as frozen aliquots. For particle stimulation, the RAW cells were incubated for 30 min at 37 °C with 3×10^6 zymosan (Molecular Probes), *S. aureus* or *S. minnesota* particles per well. After exposure to the particles, the cells were incubated for 4 h at 37 °C in

the presence of 5 $\mu\text{g ml}^{-1}$ brefeldin A to accumulate intracellular TNF- α . Where indicated, cells were exposed to 10 ng ml^{-1} LPS (*S. minnesota* R595, List), 10 $\mu\text{g ml}^{-1}$ lipoteichoic acid (*S. aureus*, Sigma) or 10 $\mu\text{g ml}^{-1}$ peptidoglycan (*S. aureus*, Fluka) with 5 $\mu\text{g ml}^{-1}$ brefeldin A for 4 h at 37 °C. After blocking Fc-receptors with 2.4G2 hybridoma supernatant, the cells were fixed with 4% paraformaldehyde in PBS. The cells were permeabilized and stained with phycoerythrin-conjugated anti-mouse TNF- α (Pharmingen) diluted in 1% fetal calf serum and 0.1% saponin in PBS. After two washes, the cells were analysed by flow cytometry using a FACScan (Beckton Dickinson) and WinMDI software (Joseph Trotter, Scripps). Brefeldin A alone did not stimulate TNF- α production (data not shown).

Phagocytosis assay

Phagocytosis by RAW cells was measured by flow cytometry of cells that had ingested fluorescently labelled particles²⁵. Zymosan, *S. aureus* or *S. minnesota* particles (3×10^6) labelled with tetramethylrhodamine were added to the macrophage monolayers and settled onto the cells by centrifugation. The cells were allowed to internalize the particles for 10 min at 37 °C, and uninternalized particles were washed away with PBS. For cells ingesting zymosan, remaining extracellular particles were dissolved by addition of 100 U ml^{-1} lyticase (Sigma) in PBS for 10 min at room temperature. The cells were resuspended in PBS + 1 mM EDTA, fixed in 1% formaldehyde, and analysed by flow cytometry. For cells ingesting bacteria, uninternalized bacteria were stripped from the cell surface by incubation in trypsin/EDTA for 5 min at room temperature, and the cells were fixed and analysed as above. It was confirmed by microscopy that the only remaining cell-associated fluorescently labelled particles had been internalized.

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Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand

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Bone remodelling and bone loss are controlled by a balance between the tumour necrosis factor family molecule osteoprotegerin ligand (OPGL) and its decoy receptor osteoprotegerin (OPG)^{1–3}. In addition, OPGL regulates lymph node organogenesis, lymphocyte development and interactions between T cells and dendritic cells in the immune system^{3–5}. The OPGL receptor, RANK, is expressed on chondrocytes, osteoclast precursors and mature osteoclasts^{4,6}. OPGL expression in T cells is induced by antigen receptor engagement⁷, which suggests that activated T cells may influence bone metabolism through OPGL and RANK. Here we report that activated T cells can directly trigger osteoclastogenesis through OPGL. Systemic activation of T cells *in vivo* leads to an OPGL-mediated increase in osteoclastogenesis and bone loss. In a T-cell-dependent model of rat adjuvant arthritis characterized by severe joint inflammation, bone and cartilage destruction and crippling, blocking of OPGL through osteoprotegerin treatment at the onset of disease prevents bone and cartilage destruction but not inflammation. These results show that both systemic and local T-cell activation can lead to OPGL production and subsequent bone loss, and they provide a novel paradigm for T cells as regulators of bone physiology.

Remodelling of bone involves the synthesis of bone matrix by osteoblasts and bone resorption by osteoclasts⁸. Excessive osteoclast activity is observed in many osteopenic disorders characterized by increased bone resorption and crippling bone damage, including post-menopausal osteoporosis⁹, Paget's disease¹⁰ and lytic bone metastases¹¹. Local or generalized bone loss has also been reported in chronic infections (hepatitis, HIV)¹², leukaemias¹³, autoimmune and allergic diseases¹⁴, and rheumatoid arthritis¹⁵, suggesting that an activated immune system can affect bone physiology. Although

Table 1 Bone mineral density

Groups	BMD in metaphysis (mg cm ⁻³)		BMD in diaphysis (mg cm ⁻³)	
	Total	Trabecular	Total	Trabecular
<i>rag1</i> ^{-/-}	363.3 ± 14.5	309.5 ± 18.5	644.4 ± 3.6	ND
<i>ctla4</i> ^{+/-} <i>rag1</i> ^{-/-}	379.6 ± 7.3	291.5 ± 15.6	626.4 ± 17.0	462.8 ± 19.6
<i>ctla4</i> ^{-/-} <i>rag1</i> ^{-/-}	302.7 ± 14.9*	209.5 ± 44.9*	529.8 ± 3.4*	353.0 ± 10.8*

Bone mineral density (BMD) was measured by peripheral quantitative computed tomography of tibial bones from *rag1*^{-/-} (*n* = 3) and *rag1*^{-/-} mice reconstituted with *ctla4*^{+/-} (*n* = 3) or *ctla4*^{-/-} (*n* = 3) bone marrow cells eight weeks after cell transfer.

* Statistically significant difference between different groups (Student's *t*-test: *P* < 0.05). Values are given as the mean ± s.d. ND, not determined.

cytokines, such as tumour necrosis factor (TNF) α , interleukin (IL)-1, IL-11 and IL-17¹⁶, that regulate immune functions have been implicated in the regulation of bone homeostasis, the molecular mechanism by which the immune system affects bone metabolism has never been established.

Consistent with previous reports^{7,17}, we observed that OPGL (also known as TRANCE, RANK-L or ODF) messenger RNA expression was upregulated in murine T cells following antigen receptor engagement (Fig. 1a). Specific inhibitor studies showed that induction of OPGL was dependent on protein kinase C (PKC), phosphoinositide 3-kinase (PI3K) and calcineurin-mediated signalling pathways (Fig. 1a). Expression of OPGL protein was detected on the surfaces of activated, but not resting, T cells (Fig. 1b). Activated T cells also secreted soluble OPGL into culture medium (Fig. 1c), and soluble OPGL was detected by enzyme-linked immunosorbent assay (ELISA) ($\sim 2.2 \pm 0.2$ ng ml⁻¹ of OPGL in the culture supernatant of CD4⁺ T cells activated for 4 days with α CD3 plus α CD28). To determine whether the secreted and/or surface forms of OPGL were functionally active, we assessed the ability of both molecules to induce osteoclastogenesis *in vitro*. Haematopoietic bone marrow precursors from wild-type mice were co-cultured with activated CD4⁺ T cells fixed with paraformaldehyde or with culture supernatants from activated T cells. Both membrane-bound and soluble OPGL supported osteoclast development *in vitro* (Fig. 1d). Osteoclastogenesis was blocked in both cases in the presence of the physiological decoy receptor osteoprotegerin (OPG) (Fig. 1d). As certain T-cell-derived cytokines can regulate OPGL expression in stromal cells, we neutralized many different cytokines including IL-1, TNF α , IL-6, interferon (INF) γ , IL-3, and granulocyte/macrophage colony-stimulating factor (GM-CSF), and none of these cytokines proved critical for *in vitro* osteoclastogenesis in this culture system, confirming that T-cell-derived OPGL is the crucial mediator. To exclude the possibility that another surface receptor on fixed T cells activates stromal cells for OPGL expression, we used spleen cells from newborn *opgl*^{-/-} mice as a source of haematopoietic precursors and fixed wild-type CD4⁺ T cells. In this culture, the only source of OPGL is membrane-bound OPGL on the T cells. Wild-type T cells induced the formation of tartrate-resistant acid phosphatase (TRAP)⁺ osteoclasts from *opgl*^{-/-} progenitor cells, and osteoclastogenesis was inhibited by addition of OPG (Fig. 1d). Osteoclastogenesis was confirmed by detection of the osteoclast-specific markers calcitonin receptor (CTR), RANK (Fig. 1e), cathepsin K (Fig. 1f) and β_3 -integrin (Fig. 1g). Thus, T-cell-triggered osteoclasts display a typical osteoclast phenotype (TRAP⁺, CTR⁺, cathepsin K⁺, β_3 -integrin⁺, RANK⁺). These results provide genetic evidence that activated T cells can directly trigger osteoclastogenesis through membrane-bound and soluble OPGL.

To evaluate whether T-cell activation and expression of OPGL affect bone physiology *in vivo*, we explored the phenotype of *ctla4*^{-/-} mice, in which T cells are spontaneously activated¹⁸. All *ctla4*^{-/-} mice displayed severe osteoporosis compared with heterozygote littermates (Fig. 2a–d). To further explore the effect of spontaneously activated T cells on bone metabolism, we adoptively transferred *ctla4*^{+/-} or *ctla4*^{-/-} bone marrow cells into lymphocyte-deficient

rag1^{-/-} mice¹⁹ and examined bone structure eight weeks after the cell graft. *rag1*^{-/-} mice have normal numbers of osteoclasts, normal bone morphology and normal bone mineral densities (data not shown). Transfer of *ctla4*^{-/-} cells into *rag1*^{-/-} mice caused a significant decrease in total and trabecular bone mineral densities compared with control *ctla4*^{+/-}*rag1*^{-/-} chimaeras and *rag1*^{-/-} mice (Table 1). Histologically, *ctla4*^{-/-}*rag1*^{-/-} chimaeric mice exhibited extended resorption of trabecular bone below the growth plates and epiphyses of the femur and tibia (Fig. 2e–h). In addition to decreased mineral density, actual bone loss was observed by histo-

morphometry (Table 2). Whereas only a few TRAP⁺ osteoclasts were detectable in *ctla4*^{+/-}*rag1*^{-/-} mice, osteoclast numbers were significantly increased in *ctla4*^{-/-}*rag1*^{-/-} mice (Table 2, and Fig. 2i, j). We observed a similar loss in bone after transfer of purified *ctla4*^{-/-} T cells into *opgl*^{-/-} mice (data not shown), implying that activated T cells can directly cause bone loss *in vivo*. Daily injection of *ctla4*^{-/-} mice with the decoy receptor OPG, which blocks OPGL action, increased bone density and significantly reduced the number of osteoclasts at the growth plates (Table 2, and Fig. 2k, l). These results show that

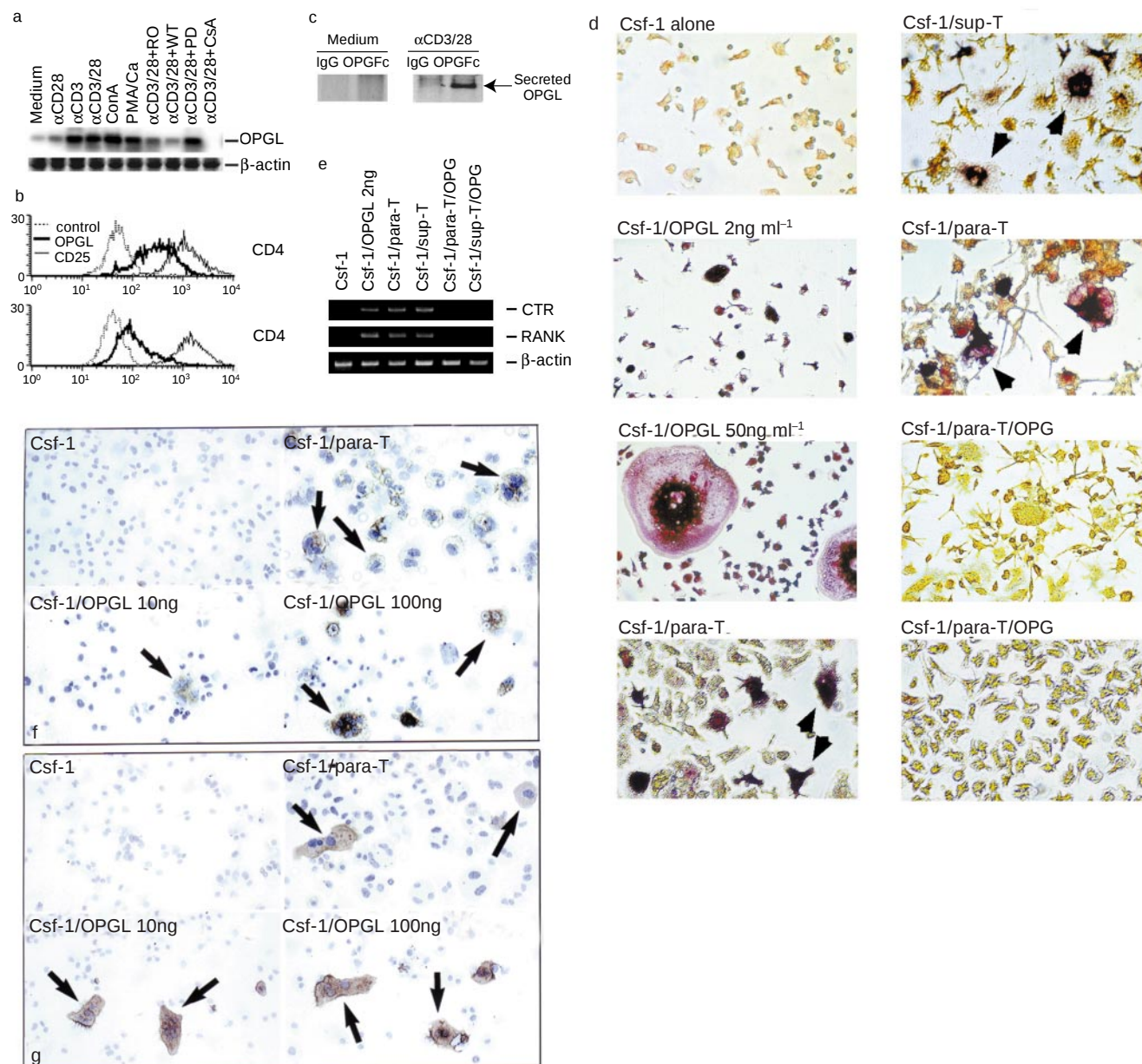


Figure 1 Activated T cells induce osteoclastogenesis through OPGL. **a**, Purified CD4⁺ lymph-node T cells were activated for 16 h with the indicated stimuli in the presence or absence of the signalling inhibitors RO, WT, PD and CsA (see Methods). OPGL and β -actin mRNA expression were detected by RT-PCR. **b**, Expression of OPGL on the surfaces of T cells stimulated with anti-CD3 ϵ plus anti-CD28 antibodies for 4 days. OPGL expression was detected by FACS. CD25 expression is shown to control for activation. **c**, Expression of secreted OPGL protein in supernatants of control T cells (left) or T cells stimulated with anti-CD3 plus anti-CD28 for 2 days (right). **d**, *In vitro* osteoclastogenesis. Bone marrow precursors from wild-type mice (top six panels) and newborn *opgl*^{-/-} mice (bottom two

panels) were cultured with Csf-1 alone; Csf-1 plus OPGL; Csf-1 plus supernatant of activated T cells (csf-1/sup-T); Csf-1 plus fixed OPGL⁺CD4⁺ T cells (csf-1/para-T); or Csf-1 plus fixed OPGL⁺CD4⁺ T cells plus OPG (500 ng ml⁻¹) (csf-1/para-T/OPG). Arrowheads show TRAP⁺ osteoclasts. **e–g**, Calcitonin receptor (CTR), RANK, cathepsin K and β_3 -integrin expression. Haematopoietic progenitors from *opgl*^{-/-} mice were cultured as above. CTR, RANK and β -actin mRNA expression were detected by RT-PCR (**e**). Expression of cathepsin K (**f**) and β_3 -integrin (**g**) was detected by immunostaining. Arrows indicate osteoclasts.

systemic activation of T cells leads to bone loss *in vivo* through OPGL.

Arthritis in humans is characterized by synovial inflammation, erosion of bone and cartilage, severe joint pain and ultimately life-long crippling¹⁵. In Lewis rats, experimental induction of adjuvant-induced arthritis (AdA) leads to severe inflammation in the bone marrow and soft tissues surrounding joints accompanied by extensive local bone and cartilage destruction, loss of bone mineral density and crippling²⁰. This condition in rats mimics many of the clinical and pathological features of human rheumatoid arthritis. Lesions in AdA are strictly dependent on T-cell activation²¹. Thus, we tested the role of OPGL in the pathogenesis of AdA arthritis in Lewis rats. OPGL protein was expressed on the surface of activated T cells isolated from rats showing clinical onset of arthritis, and OPGL mRNA could be detected in both synovial cells and inflammatory cells by *in situ* hybridization. At the onset of disease, arthritic rats were either left untreated or treated with OPG for seven days. Inhibition of OPGL through OPG had no effect on the severity of inflammation (Fig. 3a); however, OPG treatment completely abolished the loss of mineral bone density (Fig. 3b). Histologically, OPG-treated arthritic rats exhibited minimal loss of cortical and trabecular bone, whereas untreated AdA animals developed severe bone lesions characterized by partial to complete destruction of cortical and trabecular bone, erosion of the articular cartilages and clinical crippling (Fig. 4a–f). Bone destruction in untreated arthritic rats correlated with a dramatic increase in osteoclast numbers, whereas OPG treatment prevented the accumulation of osteoclasts (Figs 3c and 4e, f). These results show that OPGL is a key mediator of joint destruction and bone loss in adjuvant arthritis.

Alteration of cartilage structures leading to cartilage collapse

Table 2 Numbers of osteoclasts in the cortical bone

Groups	Number of osteoclasts in bone area	Number of osteoclasts in total area	BA%TA
<i>ctla4</i> ^{+/+}	7.69 ± 2.30	1.82 ± 0.73	23.5 ± 3.4
<i>ctla4</i> ^{-/-}	77.69 ± 23.12*	11.63 ± 3.85*	14.4 ± 1.7*
<i>ctla4</i> ^{-/-} /OPG	33.52 ± 12.18†	4.00 ± 1.37†	ND
<i>rag1</i> ^{-/-}	6.86 ± 3.43	2.58 ± 1.33	35.9 ± 5.6
<i>ctla4</i> ^{+/+} <i>rag1</i> ^{-/-}	18.81 ± 13.12	7.74 ± 6.09	33.7 ± 5.4
<i>ctla4</i> ^{-/-} <i>rag1</i> ^{-/-}	167.06 ± 32.59‡	26.43 ± 1.80‡	16.6 ± 1.7‡

Osteoclast numbers (mean ± s.e.m.; see Methods) were counted in tibial bones from *ctla4*^{+/+} (*n* = 3), *ctla4*^{-/-} (*n* = 3) and *ctla4*^{-/-} mice injected subcutaneously daily with OPG (1 mg kg⁻¹ body weight in PBS) on days 21–29 after birth; and *rag1*^{-/-} (*n* = 3) and *rag1*^{-/-} mice reconstituted with *ctla4*^{+/+} (*n* = 3) or *ctla4*^{-/-} (*n* = 3) bone marrow cells eight weeks after cell transfer. Histomorphometry was performed on cortical bone in the diaphysis of tibial bones and is shown as mean ± s.d. of bone area (BA) as a percentage of total tissue area (TA). ND, not determined.

* Statistically significant differences between *ctla4*^{+/+} and *ctla4*^{-/-} groups.

† Statistically significant differences between *ctla4*^{-/-} and OPG-treated *ctla4*^{-/-} groups.

‡ Statistically significant differences between *ctla4*^{+/+}*rag1*^{-/-} and *ctla4*^{-/-}*rag1*^{-/-} groups (Student's *t*-test; *P* < 0.05).

constitutes a critical step in arthritic joint destruction. There is controversy about whether cartilage destruction occurs independently of bone loss, or whether damage to the subchondral bone indirectly causes cartilage deterioration^{22,23}. Because OPG treatment prevented bone loss but not inflammation, we analysed the structural integrity of joint cartilage. In untreated arthritic rats, partial or complete erosion of the cartilage matrix in both the central and peripheral regions of joint surfaces was observed, as assessed by loss of toluidine blue staining (Fig. 4h). These erosions occurred in association with destruction of the underlying bony plate. In striking contrast, the integrity of cartilage was preserved in OPG-treated arthritic rats (Fig. 4i). Whether OPG protects the cartilage

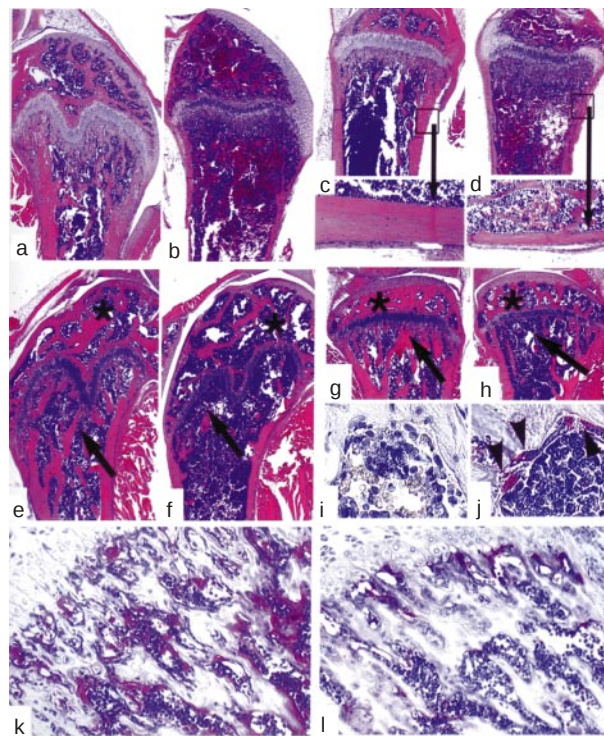


Figure 2 Activated T cells affect bone physiology *in vivo*. **a–d**, Histology of femur (**a**, **b**) and tibia (**c**, **d**) of *ctla4*^{+/+} (**a**, **c**) and *ctla4*^{-/-} (**b**, **d**) mice. Note significantly reduced thickness of cortical bone in *ctla4*^{-/-} mice. **e–h**, Histology of femur (**e**, **f**) and tibia (**g**, **h**) from *rag1*^{-/-} mice reconstituted with *ctla4*^{+/+} (**e**, **g**) and *ctla4*^{-/-} (**f**, **h**) bone marrow cells. Samples were analysed eight weeks after cell transfer. *ctla4*^{-/-}*rag1*^{-/-} mice show extended resorption of trabecular bone below the growth plate (arrows) and in

the epiphysis (asterix) of both femur and tibia. **i**, **j**, Increased number of TRAP⁺ osteoclasts in *ctla4*^{-/-}*rag1*^{-/-} (**j**) compared with *ctla4*^{+/+}*rag1*^{-/-} chimaeras (**i**).

k, **l**, OPG treatment reduces osteoclast numbers in *ctla4*^{-/-} mice. *ctla4*^{-/-} mice were injected subcutaneously daily with PBS (**k**) or OPG (1 mg kg⁻¹ body weight in PBS) on days 21–29 after birth. Note the decreased number of TRAP⁺ osteoclasts in the femur growth plate of OPG-treated *ctla4*^{-/-} mice (**l**) (TRAP, 20×). Staining: HE (**a–h**); TRAP (**i–l**).

indirectly by maintain the underlying subchondral bone or has a direct role in cartilage maintenance needs to be determined. In addition, we have found that T cells isolated from joints of all human rheumatoid arthritis ($n = 20$) and all osteoarthritis ($n = 10$) patients express OPGL (see Supplementary Information). The significance of T cell-derived OPGL in human arthritis needs to be determined. These data show that OPGL is a key regulator of bone metabolism, and that inhibition of OPGL activity by OPG can prevent cartilage destruction, a critical, irreversible step in the pathogenesis of arthritis.

Our results show that activated T cells can regulate systemic and local bone loss through OPGL. In summary, activated T cells produce OPGL and can directly trigger osteoclastogenesis *in vitro*; activated T cells from *ctla4*^{-/-} mice have a destructive effect on bone mineral density *in vivo* that can be reversed through inhibition of OPGL; and inhibition of OPGL through OPG can completely

prevent bone and cartilage loss in a T-cell-dependent arthritis model. In addition, we have found that six out of seven spontaneously arising T-cell lymphomas in mice express high levels of OPGL on T-cell surfaces, and that these lymphoma cells can directly support *in vitro* osteoclastogenesis (data not shown). As mutant mice lacking T and B cells exhibit normal bone morphology and bone mineral densities, T cells are probably not required for normal

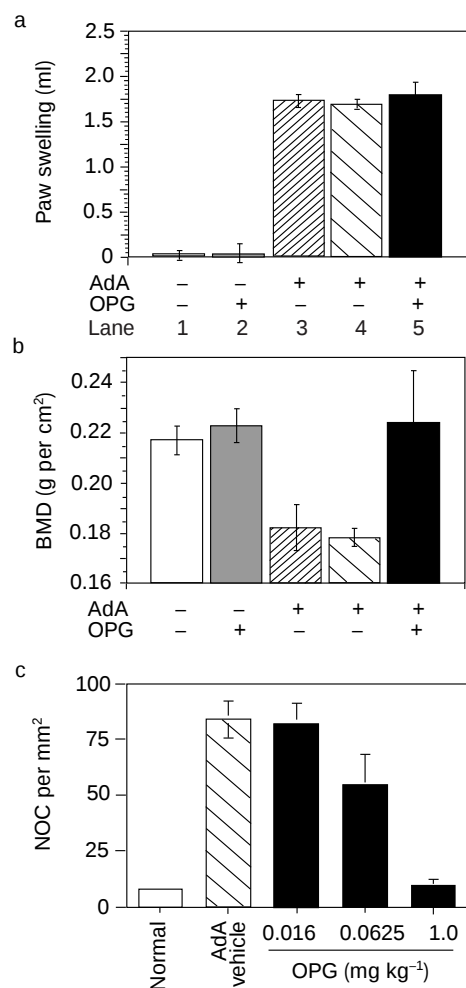


Figure 3 OPG blocks bone loss in adjuvant-induced arthritis (AdA). **a**, Severity of inflammation was calculated by measuring the volume of hind paw swelling on day 16 after initiation of disease. Lane 1, untreated controls; lane 2, OPG (1 mg kg⁻¹ day⁻¹) treatment but no AdA; lane 3, AdA but no OPG treatment; lane 4, AdA plus OPG vehicle control; lane 5, AdA plus OPG (1 mg kg⁻¹ day⁻¹). Mean values of swelling (water displacement (ml)) $\pm$ s.d. are shown ($n = 6$ per group). Paw swelling was similar between AdA and AdA plus OPG groups at all time points (days 9–15). **b**, Bone mineral density (BMD) of the tibiotarsal region was determined on day 16. Lanes are as in (a). Mean values of BMD $\pm$ s.d. are shown ($n = 6$ per group). **c**, Numbers of osteoclasts (NOC) per mm² of total bone area in the navicular tarsal bone were determined on day 16. For induction of AdA in Lewis rats and daily subcutaneous injections with soluble OPG from day 9 (onset of disease) to day 15, see Methods.

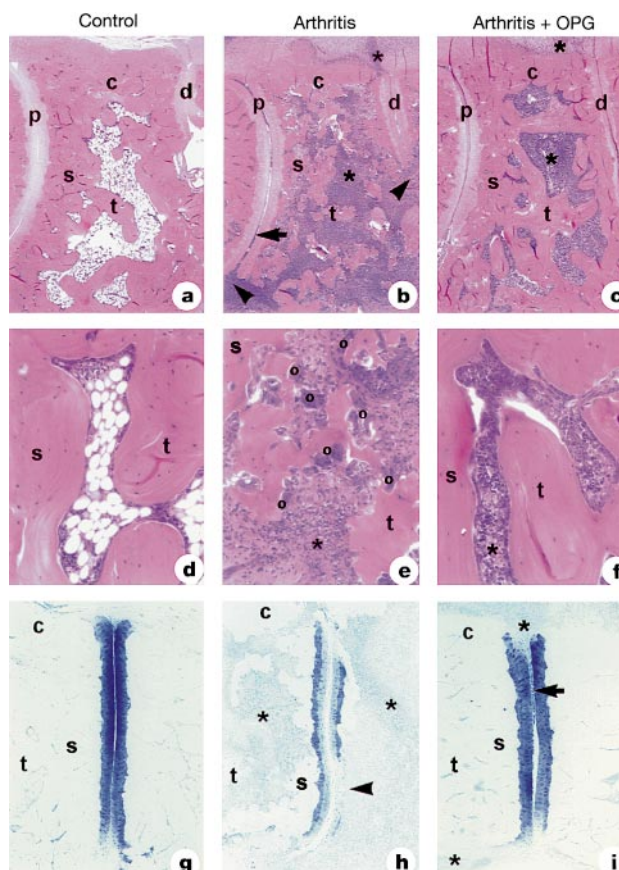


Figure 4 OPG prevents bone and cartilage destruction even in the presence of severe inflammation. **a, d**, Bone and joint structure in the normal hind paw showing dense bony plates, intact articular cartilages and marrow cavities containing scattered haematopoietic precursor cells. Proximal (p) distal (d) intertarsal joints. **b, e**, Disrupted bone and joint structure in AdA rats (day 16) with severe mononuclear infiltration in the bone marrow (asterisk), and pannus (arrow); advanced destruction (arrowheads) of cortical (c), subchondral (s) and trabecular (t) bone; and erosion of the articular cartilages. The marrow cavity contains a marked mononuclear cell infiltration (asterisk) containing numerous osteoclasts (o). These rats show severe clinical crippling. **c, f**, Preserved bone and joint structure of AdA rats (day 16) treated with OPG (1 mg kg⁻¹ day⁻¹) on days 9–15. OPG-treated rats exhibit extensive mononuclear cell infiltration of bone marrow (asterisk) and pannus formation (arrow), but cortical (c), subchondral (s) and trabecular (t) bone and articular cartilages are intact. Note the absence of osteoclasts as compared with non-OPG-treated rats (e). These rats do not show any clinical signs of crippling. **g**, Normal cartilage integrity in control rats as determined by toluidine blue staining. The matrix of normal articular cartilage is uniformly stained, and the underlying bony plates are dense. **h**, Cartilage matrix degeneration in AdA rats (day 16). Uniform pallor (small arrow) in the upper half of articular cartilages denotes extensive loss of matrix proteoglycans. Subchondral (s), cortical (c) and trabecular (t) bone is extensively eroded, and the marrow cavity is filled with inflammatory cells (asterisks). **i**, OPG treatment preserves matrix proteoglycans of arthritic rats (day 16) treated with OPG (1 mg kg⁻¹ day⁻¹) on days 9–15. Note modest reduction of toluidine blue staining in peripheral cartilage regions that are in direct contact with inflamed synovial tissue (asterisks) or pannus (arrows). Subchondral (s), cortical (c) and trabecular (t) bone is intact. Staining: HE (a–f); toluidine blue (g–i). Magnifications: 50 $\times$ (a–c); 250 $\times$ (d–f); 75 $\times$ (g–i).

bone homeostasis. Local inflammation within the bone due to metastasis, infections or fractures, or joint inflammation in arthritis, however, attracts T cells which appear to actively participate in bone remodelling through production of OPGL. To our knowledge, our results provide the first definitive linkage between activated T cells and bone homeostasis and that systemic or local activation of T cells triggers bone loss through expression of OPGL. These findings provide the molecular explanation for bone loss associated with diseases having immune-system involvement, such as adult and childhood leukaemias, autoimmunity and various viral infections such as hepatitis and HIV. Thus, inhibition of OPGL function might ameliorate many osteopenic conditions and prevent the bone destruction and cartilage damage that ultimately cause crippling in arthritis. □

Methods

T-cell activation and osteoclastogenesis

Purified ($>98\%$ CD3⁺) CD8⁺ and CD4⁺ T cells (10^6 per well) were seeded in Iscove's modified Dulbecco medium (IMDM) and stimulated with anti-CD3 ϵ ($2\mu\text{g ml}^{-1}$) and anti-CD28 (200 ng ml^{-1}) antibodies (PharMingen) in the absence or presence of the MAPK/ERK inhibitor PD98059 (PD, $4\mu\text{M}$), the calcineurin inhibitor cyclosporin A (CsA, 100 ng ml^{-1}), the PKC inhibitor RO-31-8220 (RO, $2\mu\text{M}$) or the PI3K inhibitor wortmannin (WT, $2\mu\text{M}$) for various times. OPGL mRNA expression was detected by RT-PCR as described¹⁷. Membrane-bound OPGL was detected by double staining using OPG-fluorescein isothiocyanate (FITC) and anti-CD4-PE or anti-CD8-PE. For CD25 (IL-2R α chain) expression, cells were stained with anti-CD25-FITC and anti-CD4-PE or anti-CD8-PE. Viable cells were collected by FACS analysis. For detection of secreted OPGL protein, T cells (5×10^5) were stimulated with anti-CD3 ϵ ($2\mu\text{g ml}^{-1}$) plus anti-CD28 (200 ng ml^{-1}) for 2 days and metabolically labelled with $1\mu\text{Ci ml}^{-1}$ of [^{35}S]methionine for the last 100 min of incubation. Labelled OPGL was immunoprecipitated from supernatants using either $1\mu\text{g ml}^{-1}$ of human OPG-Fc¹ or $1\mu\text{g}$ of isotype-matched control IgG. In addition, secreted OPGL protein was determined in supernatants of anti-CD3 ϵ and anti-CD28 stimulated T cells (4 days) by ELISA. OPGL expression on the surface of T cells was detectable 2 days after stimulation with anti-CD3 and anti-CD28, and maximum expression was observed at day 4 of stimulation. Throughout the whole time of the co-culture, expression of OPGL on fixed T cells was stable (data not shown). For *in vitro* osteoclastogenesis, purified CD4⁺ T cells were stimulated for 4 days with anti-CD3 ϵ plus anti-CD28 and fixed with 2.5% paraformaldehyde. Fixed T cells (10^6 cells per well) were co-cultured with non-adherent hematopoietic progenitors (10^5 cells well⁻¹) from bone marrow cells of wild-type mice or spleen cells of newborn *opgl*^{-/-} mice in the presence of 30 ng ml^{-1} colony-stimulating factor-1 (Csf-1; Genzyme) in a flat-bottom 96-well plate. The soluble decoy receptor OPG (500 ng ml^{-1}) was added to control co-cultures to confirm that the observed effects were mediated by OPGL. OPG was used as described¹. Osteoclast differentiation was evaluated on day 4 by cytochemical staining for TRAP, which specifically labels osteoclasts with a red dye, immunohistochemistry to detect Cathepsin K and β_3 -integrin, and by RT-PCR for calcitonin receptor (CTR) and RANK expression. PCR primers were β -actin sense; CACTGCCGATCCTCTTCTCTC; β -actin antisense, GCTGTGCGCTTCACCGTTCCA; calcitonin receptor sense, ACAACTGTGCTGCTGAGTG; calcitonin receptor antisense, GAAGCAGTAGATAGTCGCCAC; RANK sense, GCAACTCCAGTCAGCA; RANK antisense, GAAGTCACAGCCCTCAGAATC. To detect expression of β_3 -integrin and cathepsin K protein, *opgl*^{-/-} spleen cells were cultured with wild-type T cells for 4 days, and the expression was detected as described²⁴.

In vivo bone remodelling

ctla4^{-/-} and *rag1*^{-/-} gene-targeted mice were back-crossed onto a C57BL/6 background for at least eight generations^{18,19}. For chimaeras, bone marrow cells or purified lymph-node T cells from *ctla4*^{-/-} and *ctla4*^{-/-} littermate mice were transferred into 6-week-old irradiated (300 rad) *rag1*^{-/-} and into 6-week-old irradiated (500 rad) *opgl*^{-/-} mice. Chimaerism was monitored by FACS staining for labelled monoclonal antibodies reactive to surface markers on T cells (anti-CD4, anti-CD8, anti-TCR) or B cells (anti-CD19, anti-B220, anti-slgM) (all from PharMingen). In all experiments, chimaerism of these mice was more than 90%. RAG-chimaeric mice were necropsied 8–10 weeks after cell transfer. *ctla4*^{-/-} T cell → *opgl*^{-/-} chimaeras were necropsied 12 days after cell transfer. Bone mineral density was determined as described². Histology and histomorphometry were done as described². Osteoclast numbers were determined in a $0.5 \times 1.0\text{ mm}$ rectangle area distal to the growth plate in the tibia. The field of measurement started adjacent to the growth plate, in the centre of the marrow cavity not including the cartilage or cortical bone.

Adjuvant-induced arthritis (AdA)

Eight- to nine-week male Lewis rats were given subcutaneous (s.c.) injections of *Mycobacterium tuberculosis* (0.5 mg emulsified in 50 μl of paraffin oil) into the base of the tail (Difco Laboratories, Detroit). At the onset of clinical inflammation (day 9 after inoculation), animals were injected s.c. with soluble OPG (0.016, 0.0625 and $1\text{ mg kg}^{-1}\text{ day}^{-1}$) on each of days 9–15. On day 16, animals were killed and bone mineral

density (BMD) of the tibiotarsal region was determined by DEXAScan of the hind paws using a dual-energy X-ray absorptiometer (Hologic QDR 4500). Severity of inflammation was monitored daily on days 8–16 by measuring the volume of hind paws by water displacement. Paws were processed into paraffin as described above, and serial sections were stained with haematoxylin and eosin (HE) (to grade inflammatory and bone destructive changes) and toluidine blue (to assess the integrity of cartilage by visualizing matrix proteoglycans). Criteria for scoring the extent of inflammation and cartilage matrix integrity have been defined²⁰. Numbers of osteoclasts were counted in the navicular tarsal bone using histomorphometric measurements by tracing the section image onto a digitizing plate with the aid of a camera lucida and 'Osteomeasure' (Osteometrics Inc., Decatur, GA) bone analysis software. Two separate $560 \times 560\mu\text{m}$ regions of the talus bone were measured for each individual section. Osteoclasts included in the measurement were identified morphologically and had to be in contact with a bone surface. Experimental results are reported as the average number of osteoclasts (NOC) per mm² tissue of both left and right ankles. Animal experiments were in accordance with institutional guidelines.

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Biogen and immune disease

The immune system provides a protective mechanism through which the body defends itself against invading organisms. For centuries, scientists and physicians have sought ways to increase the body's ability to resist these pathogens, as well as to solve the riddles of diseases that occur when the body turns its immune system against itself. While there have been many successes, the problems of infectious and autoimmune diseases remain among the most profound unsolved questions in medicine.

The revolution in genetic engineering has provided important new approaches to solving these problems, through intensive study in both academic and pharmaceutical laboratories worldwide, including Biogen, which has a long and distinguished history of research into immune disease. Founded in 1978 by an international group of distinguished scientists, two of Biogen's early discoveries are among the world's leading pharmaceutical products. They are INTRON® A alpha interferon, marketed throughout the world by Schering-Plough Corporation, for treatment of hepatitis B and C and certain cancers, and the hepatitis B vaccines, sold by SmithKline Beecham and Merck. In 1996, Biogen received licensure to market AVONEX® (Interferon beta-1a), which has become the world's leading treatment for relapsing forms of the autoimmune disease multiple sclerosis. Biogen has maintained its strength in this arena of biology, and is investigating multiple molecules and molecular pathways relevant to autoimmune and allergic diseases. These include members of the TNF family, such as CD40 ligand and lymphotoxin beta, the integrins, particularly VLA4 and its interaction with VCAM1, the CD2/LFA3 pathway, and, of course, the interferons.

Biogen believes that continued fundamental research in immunology will be of central importance to our understanding of human disease. Few pathologies are unaccompanied by inflammatory and immune responses. The rapid pace of advances in this area, combined with novel technologies for drug discovery and the sequencing of the human genome, promise to produce an unprecedented increase in the pace of drug discovery in the first years of the new millennium. As such, we are proud to sponsor this supplement, which showcases some of the key advances made in the last year in our understanding of both innate and acquired immune responses.

Joseph M. Davie, MD PhD, Senior Vice President—Research

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